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# CONTENTS

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## No. 1. JANUARY 5

EDWIN G. CONKLIN. Cell size and nuclear size. Thirty-seven figures.....	1
RAYMOND PEARL AND MAYNIE R. CURTIS. Studies on the physiology of reproduction in the domestic fowl. V. Data regarding the physiology of the oviduct. Four figures.....	99
L. B. NICE. Comparative studies on the effects of alcohol, nicotine, tobacco smoke and caffeine on white mice. I. Effects on reproduction and growth. One figure.....	133

## No. 2. FEBRUARY 10

FRANK W. BANCROFT. Heredity of pigmentation in <i>Fundulus</i> hybrids. Thirty figures.....	153
PHIL RAU AND NELLIE RAU. Longevity in Saturniid moths; an experimental study. Five figures.....	179
LORANDE-LOSS WOODRUFF. Observations on the origin and sequence of the protozoan fauna of hay infusions. Fifteen figures.....	205
MORRIS S. FINE. Chemical properties of hay infusions with special reference to the titratable acidity and its relation to the protozoan sequence. Five figures.....	265
A. FRANKLIN SHULL. Studies in the life cycle of <i>Hydatina senta</i> . III. Internal factors influencing the proportion of male-producers. Six figures.....	283

## No. 3. APRIL 5

HELEN DEAN KING. Studies on sex-determination in Amphibians. V. The effects of changing the water content of the egg, at or before the time of fertilization, on the sex-ratio of <i>Bufo lentiginosus</i> .....	319
DAVID DAY WHITNEY. Reinvigoration produced by cross fertilization in <i>Hydatina senta</i> .....	337
MANTON COPELAND. The olfactory reactions of the puffer or swellfish, <i>Spheroides maculatus</i> (Bloch and Schneider).....	363
JOHN C. PHILLIPS. Size inheritance in ducks.....	369
JACQUES LOEB AND F. W. BANCROFT. Can the spermatozoön develop outside the egg? Eleven figures.....	381
DAVID H. TENNENT. Studies in cytology. I. A further study of the chromosomes of <i>Toxopneustes variegatus</i> . II. The behavior of the chromosomes in <i>Arbacia-Toxopneustes</i> crosses. Twenty-one figures.....	391

## No. 4. MAY 20

FRANK R. LILLIE. Studies of fertilization in <i>Nereis</i> . III. The morphology of the normal fertilization of <i>Nereis</i> . IV. The fertilization power of portions of the spermatozoön. Forty-eight figures.....	413
T. H. MORGAN. The elimination of the sex-chromosomes from the male-producing eggs of <i>Phylloxerans</i> . Twenty-nine figures.....	479
A. H. STURTEVANT. An experiment dealing with sex-linkage in fowls. Four figures.....	499
MERKEL HENRY JACOBS. Studies on the physiological characters of species. I. The effects of carbon dioxide on various protozoa.....	519
JACQUES LOEB AND HARDOLPH WASTENEYS. On the adaptation of fish ( <i>Fundulus</i> ) to higher temperatures.....	543

# CELL SIZE AND NUCLEAR SIZE

EDWIN G. CONKLIN

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THIRTY-SEVEN FIGURES

## CONTENTS

PART I. CELL SIZE AND NUCLEAR SIZE IN NORMAL DEVELOPMENT.....	4
I. Unequal cell division.....	4
1. The maturation divisions.....	4
2. Cleavage.....	6
3. Significance of the yolk lobe.....	9
II. Cell size and nuclear size in eggs and blastomeres.....	12
1. Cell size and nuclear size in the cleavage of <i>Crepidula plana</i> .....	14
2. Cell size and nuclear size in the cleavage of <i>Fulgar carica</i> .....	23
III. Cell size and nuclear size in adult tissue cells.....	25
IV. The inciting causes of cell division.....	29
V. Growth of protoplasm during cleavage.....	32
VI. Rate of nuclear growth during cleavage.....	36
1. Nuclear growth during the cleavage of the egg of <i>Crepidula</i> .....	38
2. Nuclear growth during the cleavage of the egg of <i>Fulgar</i> .....	40
3. Nuclear growth during the cleavage of other animals.....	42
4. Growth of different nuclear constituents.....	44
a. Nuclear sap.....	44
b. Linin.....	46
c. Chromatin.....	47
d. Chromosomes.....	48
e. Plasmasomes.....	51
f. Centrosomes and spheres.....	53
5. Conclusions as to nuclear growth during cleavage.....	54
6. Comparison of growth of chromatin with increase of chemical substances and processes during cleavage.....	56
VII. Senescence, rejuvenescence, and the ratio of nucleus to plasma.....	57
PART II. EXPERIMENTAL STUDY OF CELL SIZE AND NUCLEAR SIZE IN THE EGGS OF CREPIDULA PLANA.....	63
I. Nuclear size and chromosome number.....	63
II. Nuclear size and cell size in centrifuged eggs of <i>Crepidula</i> .....	64

III. General results of these experiments .....	75
1. Nuclear size in centrifuged eggs .....	75
2. The sizes of spindles, centrosomes, spheres and astersf .....	77
3. The rhythm of division in centrifuged eggs .....	77
4. Growth of cytoplasm at the expense of yolk .....	77
5. Unequal and differential cell divisions .....	78
6. Regulation in the cleavage process .....	81
General summary and index .....	83
Literature cited .....	88

In the development of all organisms considerable differences of size appear, sooner or later, among constituent cells; sometimes the blastomeres of the cleaving egg differ in size, in other cases these differences appear only later during the blastula, gastrula, or larval stages. Endoderm cells are usually larger than those of the ectoderm, ciliated cells are generally larger than non-ciliated ones, muscle and nerve cells are usually larger than epithelial or mesenchyme cells.

These differences in the size of cells may be due to unequal cell division, to unequal rate of division, or to unequal growth of cells after division, and in some cases all of these factors may be represented in the same egg or embryo. It is frequently assumed that unequal cell divisions are caused by the accumulation of metabolic substances, such as yolk at one side of a cell, and the crowding of the protoplasm and nucleus to the opposite side. Such unequal divisions are frequently found in yolk-laden eggs, and may be artificially produced at will by centrifuging the yolk to one pole or the other of a dividing cell. But in many cases this is not the cause of unequal cell division; the yolk may be uniformly distributed with regard to the poles of the spindle and yet the cleavage may be unequal, or unequal division may take place in purely protoplasmic cells, in which the eccentric position of the spindle is not due to pressure. Innumerable cases of this sort have been found both in normal and in experimentally altered conditions. Often such unequal divisions are associated with visible histological differences in the resulting cells. The study of cell-lineage has shown that in some cases a particle distinguished from the time of its formation by its size, protoplasmic structure, rate of division, prospective significance and

potency. In such cases differences in the sizes of cell are associated with some of the earliest differentiations of the developing egg.

But differences in the size of cells may be due, not to unequal cell division, but to unequal rates of division, or to unequal growth of cells subsequent to division. In some instances cells divide rarely and consequently become large, while adjoining cells divide frequently and therefore remain small. The fact that cells are not always of the same size at the time of division is one of capital importance for it shows conclusively that the factors which bring about cell division may be separated from those which cause growth.

In connection with the size of cells as a whole may be considered the sizes of many of their constituent parts, such as nuclei, chromosomes, plasmasomes, centrosomes, etc. The size relations which exist between these parts of the cell and the plasma should throw light upon the interrelation between these cell constituents in other respects than size. The quantitative relations of different cell constituents at various phases of activity should be of significance in the study of many fundamental problems of growth, differentiation and cellular physiology.

Within the past few years several contributions on this subject have appeared, principally from Boveri and R. Hertwig, and their students. In so far as these works have dealt with the development of the egg they have been based on a study of eggs of 'indeterminate cleavage' in which it is not possible to trace individual blastomeres throughout the cleavage period; the results have therefore been mass results, based on averages of cells of a given stage. In the study of vital phenomena it is frequently important to deal with individual rather than with average results; in the following pages I have attempted to apply the method of the quantitative study of cells and of cell constituents to individual blastomeres at various stages of the cleavage.

## ART I

## CELL SIZE AND NUCLEAR SIZE IN NORMAL DEVELOPMENT

## I. Unequal cell divisions

1. *The maturation divisions.* The most unequal of all cell divisions are those which give rise to the polar bodies. The actual diameter of the first polar body and of the egg, and the relative volumes of the two, are here given for a number of different animals. These measurements were made on eggs which had been fixed, stained, and mounted in balsam.

TABLE I  
*Sizes of polar bodies and eggs*

SPECIES	DIAMETER FIRST POLAR BODY	DIAMETER OF EGG	RELATIVE VOLUMES
	$\mu$	$\mu$	
Cumingia tellinoides.....	6	45	1 : 421.8
Amphioxus lanceolatus.....	6	108	1 : 5832.0
Cynthia partita.....	9	105	1 : 1560.8
Crepidula planif.....	12	136	1 : 1442.8
Crepidula fornicata.....	12	182	1 : 3443.0
Crepidula convexa.....	15	280	1 : 6434.8
Crepidula adunca.....	15	410	1 : 20123.6
Fulgur carica.....	15	1600	1 : 1211355.0

In many other cases, such as the eggs of selachians, amphibians and birds, the disproportion between the polar body and the egg is much greater than in the cases here measured. The significant thing here is not merely the degree of inequality, but also the relative uniformity in size of the polar bodies as compared with the egg. Although the eggs of different animals vary enormously in size, the polar bodies vary relatively little, and it is safe to conclude, both from observation and experiment, that the polar bodies are in general the smallest cells which can be formed from egg cells by the process of normal cell division.

In spite of this very great inequality of the daughter cells, the mitotic figure in the first maturation division of *Crepidula* and of many other animals is the largest in the whole life cycle. When

first formed this spindle lies near the middle of the egg, and if the division wall were to form while the spindle lies in this position a polar body would be formed whose diameter would be to that of the egg as 1 : 1, 1 : 2, or at the least 1 : 3. Later the spindle moves toward the periphery until one pole comes into contact with the cell membrane. The membrane then protrudes over this pole and into this protrusion the end of the spindle moves; at the same time the spindle itself constantly grows shorter, until finally the spindle is but little more than double the diameter of the polar body, and in the separation of the polar body the division wall passes through the equator of the spindle. In *Crepidula plana* the first maturation spindle shortens to about half its original length; during the metaphase its maximum length is about  $42\mu$ , at the time when the polar body is being separated it is only  $24\mu$  long.

By means of centrifugal force it is possible to prevent the spindle from moving from its first position and also from shortening, and under these circumstances giant polar bodies are formed, sometimes quite as large as the remainder of the egg. In all such cases the division wall passes through the equator of the spindle. Evidently the factors which bring about this most unequal of all cell divisions are (1) the eccentricity and (2) the shortening of the maturation spindle.

The second polar body is but slightly smaller than the first, nevertheless the spindle is much smaller, its maximum length in *Crepidula plana* being  $18\mu$ ; correspondingly it shortens much less in the anaphase than the first polar spindle, being almost as long when the division wall begins to form as in the metaphase. Though the second polar spindle may appear at some distance from the point at which the first polar body was formed, and although its axis may lie at right angles to that of the first polar spindle, it invariably rotates into the axis of the latter and the whole spindle moves toward the surface until its outer pole comes to lie immediately under the first polar body, and here the second polar body is pushed out. In this case the principal factor which causes the inequality of division is the eccentricity of the spindle. If the spindle is prevented by pressure or cen-



trifugal force from taking this eccentric position the resulting cell division may be nearly equal, or a giant second polar body may be formed (fig. 11).

2. *Cleavage.* The first cleavage of *Crepidula* and of *Fulgur* is approximately equal. The pronuclei lie near the animal pole of the egg, the egg nucleus lying somewhat nearer the polar bodies than the sperm nucleus. The first cleavage spindle is oriented so as to lie at right angles to the egg axis, but it is impossible in these eggs to determine whether the spindle lies in a particular cross axis or not. However in typical cases the spindle invariably lies at right angles to the chief axis with its equator in that axis, and the protoplasm and yolk are divided by the first cleavage plane with strict equality. The same is true of the second cleavage which in all these regards resembles the first.

It has been generally assumed that equal cleavages, alternately at right angles, are due to simple mechanical conditions, such as the greatest diameter of the protoplasmic mass, and that they require no further explanation. As a matter of fact equal cleavages, and successively alternating ones, cannot be explained in so simple a manner. The fact that the first cleavage spindle invariably stands at right angles to the chief axis of the egg and with its equator in that axis shows that there is here some orienting power of the highest significance. It is well known that there is considerable variation in the path which the spermatozoon takes through the egg, and in its manner of meeting with the egg nucleus; there is also much variation in the actual positions of the cleavage centrosomes and in the initial position of the first cleavage spindle, without any corresponding variation in the final position of the spindle or of the cleavage plane. As a result of the study of large numbers of eggs of many different animals, under both normal and experimental conditions, it seems to me necessary to conclude that the same factor which brings about an unequal division of an egg such as that of *Unio*, operates to cause the equal division of an egg like that of *Crepidula*; this factor is to be found in the polarity and symmetry of the egg itself. In *Unio*, where the first cleavage is very unequal,

Lillie ('01) has shown that the spindle oscillates in the cell before coming to rest in its eccentric position; and in many cleavages in *Crepidula* the spindle may at first lie out of its normal position and may move later into its proper place; and this applies not only to the eccentricity but also to the axial position of the spindle. Something outside of the spindle itself determines the position which it shall take in the cell, and this is as true of equal and alternating cleavages as of unequal and non-alternating ones.

TABLE 2  
*Sizes of macromeres and micromeres in Crepidula and Fulgur*

SPECIES	MACROMERES	DIAMETER	MICROMERES	DIAMETER	RELATIVE VOLUMES
		$\mu$		$\mu$	
<i>C. plana</i> .....	1A-1D	81	1a-1d	30	ca. 19.9 : 1
	2A-2D	80	2a-2d	36	ca. 10.6 : 1
	3A-3D	76	3a-3d	33	ca. 12.1 : 1
	4D	66	4d	38	ca. 4.9 : 1
	4A-4C	60	4a-4c	42	ca. 2.7 : 1
	5A, 5B	75	5a, 5b	60	ca. 1.9 : 1
	5C, 5D	68	5c, 5d	68	ca. 1 : 1
<i>C. convexa</i> ....	1A-1D	195	1a-1d	69	ca. 22.0 : 1
	2A-2D	195	2a-2d	50	ca. 59.3 : 1
	3A-3D	195	3a-3d	50	ca. 59.3 : 1
<i>Fulgur carica</i> ..	1A-1D	800	1a-1d	80	ca. 1000 : 1
	2A-2D	800	2a-2d	80	ca. 1000 : 1
	3A-3D	800	3a-3d	80	ca. 1000 : 1
	4D	780	4d	130	ca. 216 : 1
	4A-4C	740	4a-4c	370	ca. .8 : 1

The third, fourth, and fifth cleavages of the macromeres of *Crepidula* and of other gasteropods are successively alternating in direction, and are notably unequal in the size of the daughter cells; while the sixth and all subsequent divisions of the macromeres are more nearly equal than the preceding ones. The diameters of the cells formed by these cleavages and their approximate ratios, in *Crepidula* and *Fulgur*, are shown in Table 2.

In the structure of the macromeres there is no visible organization which would explain why the first two cleavages of the egg are equal, the three following ones very unequal and subse-

quent cleavages more nearly equal again, and yet it is certain that some such organization must be present. It is generally believed that the inequality of macromeres and micromeres is due to the quantity of yolk contained in the former and where the quantity of yolk is extremely great, as in *Fulgur*, this is undoubtedly one of the causes of the great difference in the sizes of the macromeres and micromeres; but that it is not the only cause of the inequality is shown by experiments in which by centrifuging eggs at the first or second cleavage two of the macromeres come to contain no yolk, while the other two contain all of the yolk; in the macromeres which are purely protoplasmic and contain no yolk the subsequent cell divisions are still unequal, protoplasmic micromeres of the usual size being separated from the protoplasmic macromeres, (see p. 81). The study of normal as well as of artificially altered cleavage points unmistakably to the conclusion that the position and axis of each spindle is fixed by the structure of the cell protoplasm, and since the position and axis of the spindles change regularly in successive divisions this protoplasmic structure must change regularly in successive cell generations. Boveri ('05) says that the position of the spindle is not due to a permanent cell structure, but that the constitution of the egg undergoes progressive alterations, which then react on the division centers.

Among the micromeres certain cell divisions are quite unequal, and here there can be no question that this inequality of division is in no way associated with the presence of yolk, since the micromeres are purely protoplasmic. In *Crepidula* the first and second subdivisions of the first quartet cells (figs. 3, 8), which give rise respectively to the 'turret' cells and the 'apical rosette' cells, are very unequal; as is also the division of the second quartet cells which give rise to the 'tip' cells of the arms of the ectodermal cross. The diameters of the two daughter cells in each of these divisions, and the approximate ratio of one to the other, are as follows in *Crepidula plana*:

$1a^{1-1}d^1$ , $25\mu$ , $1a^{2-1}d^2$ , $13\mu$ .....	Ratio 2 : 1
$1a^{1-1-1}d^{1-1}$ , $30\mu$ , $1a^{1-2-1}d^{1-2}$ , $18\mu$ .....	Ratio 5 : 3
$2a^{1-1-1}d^{1-1}$ , $30\mu$ , $2a^{1-2-2}d^{1-2}$ , $15\mu$ .....	Ratio 2 : 1

In the case of normal eggs it cannot be demonstrated that the inequality may not be due to mutual pressure among the cells, but in certain experiments which will be described in another paper, this factor may be entirely eliminated, isolated blastomeres showing the same inequalities of division as do those in the cell complex. In all of these cases of definite types of cleavage, the position of the spindle, and consequently the direction of division and the relative size of the daughter cells, is determined by some structural peculiarity of the protoplasm and not by the presence of metabolic substances within the cell or by pressure from without.

3. *Significance of the yolk lobe.* Under normal conditions the line of intersection of the first and second cleavage planes marks the chief axis of the egg, its two ends being the animal and vegetal poles. In eggs in which the cleavages are unequal, the chief axis, thus defined, runs from the animal pole, which is marked by the position of the polar bodies, to a point more or less removed from the diametrically opposite pole.

Is this chief axis predetermined in the egg or is it established by the positions of the first and second cleavage planes? Observation alone affords no positive answer to this question, but the fact that the spindle takes a definite and characteristic position in the egg indicates that something outside the spindle determines its position, and points to the conclusion that the chief axis is already present in the egg, as a structural differentiation before cleavage begins. This conclusion is well supported by experiment, as will be shown later.

In this connection the significance of the so-called 'yolk lobe' is interesting. As is well known this lobe is found in many eggs, especially in those in which the first and second cleavages are unequal. It is present however in minute form in such eggs as those of *Crepidula* and *Fulgur* in which the first two cleavages are approximately equal, but in cases in which these cleavages are unequal it is much larger, and in general the size of the yolk lobe is proportional to the inequality of division. In all cases so far as I am aware the yolk lobe lies diametrically opposite the animal pole, and if detached from the egg at the time when it is fully formed, the egg divides into equal blastomeres, as Wilson

('04) found in *Dentalium*; if it remains attached it fuses, at the close of the cleavage, with one of the cells, which then becomes larger than the other one. In this case the cleavage spindle is not eccentric and the furrow cuts down through the center of the egg until it reaches the yolk lobe when it turns to one side of the lobe leaving it attached to one of the cells. In this way a cleavage which began as an equal one becomes unequal. Where the spindle is eccentric from the start and the furrow does not pass through the center of the egg the yolk lobe is not prominent. In this way inequality of division may arise through the eccentricity of the spindle, or through the formation of a yolk lobe which remains connected with one of the two daughter cells, which would otherwise be equal.

One cannot study the eggs of different animals without being much impressed with the fact that the distribution of yolk to the four macromeres is highly characteristic of different species and orders. Thus among prosobranchs the yolk is distributed either equally to all the macromeres, as in *Crepidula*, *Fulgur*, *Trochus*, etc., or if one of the macromeres is larger than the other three it is the left posterior macomere, *D*, as in *Nassa*, *Urosalpinx*, *Tritia*, etc. Among opisthobranchs, if the macromeres are unequal in size it is one or both of the anterior ones, *A* or *B*, which is the larger. Among pulmonates, so far as I recall, the macromeres are always equal in size.

The fact that there are these characteristic differences in the sizes of the macromeres of different orders indicates that they have some characteristic cause; and the fact that in nearly related species the macromeres may be equal or unequal indicates that in this case the cause is not a very general one. If one considers that the first and second cleavages normally pass through the egg axis, and that their position is determined by this structural feature, the unequal distribution of yolk to the four macromeres may be due to the localization of the yolk in different parts of the ovarian egg,—on the posterior side of the chief axis in prosobranchs, on the anterior side in opisthobranchs; while a larger or smaller yolk lobe would determine the degree of inequality of the macromeres in the different species.

Apart from the relation of the yolk lobe to unequal cleavage Wilson ('04) has shown that it bears some relation to the formation of the pretrochal region in the larva of *Dentalium*; when the lobe was removed the pretrochal organs failed to develop. What the morphogenetic factors are, which are located in the yolk lobe, is not known, but the significance of the lobe can scarcely be for the formation of the pretrochal region, since in animals with no lobe or with a very minute one these regions form quite as well as in those with a large lobe.

These explanations refer to the "prospective significance" of the yolk lobe, and I know of no certain evidence as to the cause of its formation. The fact that such a lobe is present in almost all gasteropod eggs, differing only in size in different species, and that it is present in the eggs of annelids and a large number of other animals, indicates that it has some cause of general occurrence. In 1897 I suggested that the yolk lobe marks the point of attachment of the ovarian egg to the follicular wall. At this point there is left a little mass of protoplasm on the surface of the egg, and here there is a weak spot in the protoplasmic pellicle which surrounds the egg. If the egg is put under pressure the yolk may be caused to flow out at this point, and in the increased tension which accompanies mitosis a yolk lobe is often pushed out at this spot.

On the whole then, it seems probable that the yolk lobe represents a temporary extrusion of egg substance during mitotic pressure at the former point of attachment to the ovarian wall, and that as a result of the presence of a large lobe of this kind, the first and second cleavages may be rendered unequal though the intersection of the furrows may lie in the egg axis and in the polar diameter of the egg.

In this connection one recalls the 'Dotterball' and the 'Granulaball' observed by Hogue ('10) and Boveri ('10) in centrifuged eggs of *Ascaris*. Boveri comes to the conclusion that these are formed because they lie outside the influence of the asters or spheres: "Man könnte vielleicht sagen:—der von einer Sphäre eingenommene Plasmabezirk sucht sich von allem was ausserhalb dieses Wirkungskreises liegt, abzuschneiden," (p. 123). He sup-

poses that when the spindle, lying at right angles to the egg axis, is pushed far toward the animal or vegetal pole, a 'ball' is formed at the opposite pole. Whether this 'ball' is homologous with the yolk lobe I shall discuss in another paper in which the artificial production of such 'balls' will be considered, but I wish to point out here that although the first and second cleavage spindles in the large eggs of *Crepidula* and *Fulgur* lie near the animal pole, and far from the vegetal, the yolk lobe in these forms is very small, whereas in the minute eggs of the oyster and the clam, where the spindles are much nearer the vegetal pole, the yolk lobe is relatively very large. If, as I believe, this lobe is the result of an unsymmetrical distribution of yolk and egg substance with reference to the egg axis, or in the case of *Ascaris* with reference to the normal division plane, the great size of the lobe in some cases and its minute size in others, in which the area lying outside the "Wirkungskreise" of the spheres is much greater than in the former, would find a ready explanation.

## II. *Cell size and nuclear size in eggs and blastomeres*

Strasburger ('93) was the first to show by detailed measurements that a fairly definite ratio exists between the nuclear size and the cell size in the embryonic cells of any given species of plant. He gives tables of measurements of the sizes of nuclei and cells in some forty different species, the nuclei ranging in diameter from  $16\mu$  to  $3\mu$ , and the cells from  $24\mu$  to  $5\mu$ . In general he found that the ratio of nuclear diameter to cell diameter is approximately as 2 to 3; and the ratio which exists in any case, is held to be due in general to the 'working sphere of the nucleus,' i.e., to the extent to which the metabolic interchange between nucleus and cytoplasm can reach.

Gerassimoff ('01, '02) found, in the cell division of *Spirogyra*, that when both daughter nuclei were caused to remain in one of the daughter cells, that cell grew to a larger size than normal, and he therefore concluded that the nuclear size determines the cell size.

Boveri ('02, '05) found that the size of the nuclei in sea urchin larvae is dependent upon the number of chromosomes which enter into the nuclei; in parthenogenetic or hemikaryotic eggs the nuclei are smaller than in fertilized (amphikaryotic) ones, and they are smaller in the latter than in diplokaryotic eggs in which the number of chromosomes is greater than normal. Furthermore he found that nuclei with a small number of chromosomes are not only smaller than those containing a larger number but that the cells in which they lie are also smaller, owing to the occurrence of a larger number of cell divisions in cells with small nuclei than in cells with large ones.

Boveri's work was based primarily on his studies of echinoderm development and some of his conclusions are not applicable, without modification, to the eggs and larvae of other forms, especially forms in which there are great inequalities of cleavage and in which various cells of the larva differ markedly from one another in size. Thus his generalization, sometimes mentioned as 'Boveri's Law,' viz., "Die Grösse der Larvenzellen ist eine Funktion der in ihnen enthaltenen Chromatinmenge, und zwar ist das Zellvolumen der Chromosomenzahl direkt proportional," could not apply, without modification, to eggs or larvae in which various cells differ greatly in size without any corresponding difference in the number of chromosomes. Unequal cell divisions are frequently found in the development of mollusks, annelids and ascidians, where purely mechanical causes, such as mutual pressure between cells or the pressure of yolk within cells are not involved; in such cases the sizes of the nuclei invariably become proportional to that of the plasma, though the number of chromosomes remains the same in every nucleus. Similarly, many cells at first equal in size become unequal through dissimilar growth, and their nuclei then become unequal also. Finally, in each of the animal groups named, cells at first equal in size may become unequal through dissimilar rates of division. In all such cases the number of chromosomes appears to be, and presumably is, the same in every nucleus of a given egg or embryo. Evidently in cases of normal development the number of chromosomes does not determine the varying sizes of cells and nuclei.



R. Hertwig ('03, '08) as a result of his earlier work ('89) upon protozoa, has laid especial emphasis upon the fundamental significance of the ratio of nuclear size to cell size. He says ('03, p. 56):

Wir haben im vorhergehenden sehr komplizierte Wechselwirkung zwischen Kern und Protoplasma kennen gelernt. Verkleinerung der Kernmasse führt zu Verkleinerung der Zellgrösse (Boveri), Vergrösserung der Kernmasse zu einer Vergrösserung der Zelle (Gerassimoff, Boveri). Andererseits kann aber auch Schwund des Plasmas zu einer Reduktion des Kernmaterials Veranlassung werden. Diese Verhältnisse kann man nur erklären, wenn man die oben vertretene Annahme macht, das jeder Zelle normalerweise eine bestimmte Korrelation von Plasma- und Kernmasse zukommt, welche wir kurz die "Kernplasma-relation" nennen wollen.

More recently Hertwig and his students have made many notable contributions to these "new problems of the cell theory," as Hertwig ('08) calls them. It has long been known that large cells have large nuclei, small cells small nuclei:

Das Neue, welches in der Lehre von der Kernplasma-Relation gegeben ist, ist der Gedanke, dass das Massenverhältnis von Kern zu Protoplasma, der Quotient  $k/p$ , d.h. Masse der Kernsubstanz dividiert durch Masse des Protoplasma, ein gesetzmässig regulierter Factor ist, dessen Grösse für alle von Kerne beeinflussten Lebensvorgänge der Zelle, für Assimilation und organisierende Tätigkeit, für Wachstum und Teilung, von fundamentaler Bedeutung ist.

Hertwig calls attention to the fact that the Kernplasma-Relation differs in different phases of cell life, and he chooses for measurement that phase when the cell has come out of division and begins to nourish itself and to grow. This condition is known as the Kernplasma-Norm, and departures from it constitute what he calls Kernplasma-Spannung. This work of Hertwig and his school will be discussed more fully after the presentation of my observations.

1. *Cell size and nuclear size in the cleavage of Crepidula plana.* In my work on Karyokinesis and Cytokinesis in *Crepidula* ('02) I showed that the sizes of nuclei, spheres and asters, centrosomes, chromosomes, and plasmasomes are correlated with the quantity of cytoplasm in the cell, and the following pages constitute an

elaboration and further extension of that work. The egg of *Crepidula plana* is a particularly favorable object for the study of such a subject. The eggs may be stained and mounted entire in such manner that all of these cell constituents show with great distinctness, and the advantage of seeing whole eggs and nuclei in making such measurements is sufficiently obvious.

A further advantage of the study of whole eggs is found in the fact that the exact stage in the cell cycle is more easily determined in whole eggs than in sections. My work has shown that it is most important in comparing the sizes of cell constituents to compare precisely corresponding stages, and accordingly I have chosen for measurement stages of the maximum and minimum sizes of the nuclei. The growth of the nucleus is more rapid in the last stage of the resting period preceding mitosis ('Kernteilungswachstum' of Hertwig) than at any other time in the cell cycle, and in order to find the maximum nuclear size it is necessary to measure the nuclei just before the nuclear membrane disappears. Such stages are easily selected by looking for eggs in which part of the nuclei of a certain generation of cells are dividing while others have not yet begun to divide, as in figs. 1 and 2. At this stage there is great uniformity in the dimensions of the nuclei of particular blastomeres, and as the nuclei at this stage are regular spheres, it is easy to calculate their volumes.

The cell dimensions are more difficult to determine than are those of the nucleus. In cells which contain yolk and in cells of irregular shape it is not possible to determine the volume of the plasma with accuracy. After the first cleavage the plasma and yolk are sufficiently well separated so that the dimensions of the cytoplasm can be fairly well observed; before the first cleavage the plasma is so mixed with the yolk that this can not be done and I have here had recourse to the method of centrifuging the yolk out of the egg, leaving only the nucleus and plasma which can then be easily measured. Wherever it could be done, I have chosen cells for measurement which were as nearly spherical as possible, but where the dimensions in different axes differed considerably I have determined the mean diameter, which is the one recorded.

It is well known that during mitosis the general surface tension of a cell increases, and the cell tends to become spherical in shape. In measuring the maximum cell size, I have usually taken the stage immediately after the nuclear membrane disappears, and when the cell approaches a spherical shape. Similarly the minimum cell size has been determined by measuring the daughter cells during the telophase when they are approximately spherical. I have confidence in the substantial accuracy of my measurements of these maximum and minimum sizes of the purely protoplasmic micromeres. The volume of plasma in the yolk-containing macromeres is merely an approximation.

All measurements were made with Zeiss 1/1 micrometer eyepiece and 3 mm. homogeneous immersion objective. In all cases enough cells and nuclei were measured to give a fair average, though there is relatively little variation in the sizes of particular cells and cell constituents at corresponding stages in the cell cycle. All the eggs studied were fixed, stained and mounted in the same manner, so that alterations due to shrinkage should be approximately the same in all.

It is evident from table 3 that while large cells have larger nuclei than small cells, the relation of nuclear volume to cell volume is not constant. In different blastomeres of the same egg the Kernplasma-Relation, measuring nuclei and cells at their maximum size, varies from 1 : 14.5 to 1 : 0.37; even in purely protoplasmic cells it varies from 1 : 14.5 to 1 : 8.7. In cells containing yolk the ratio of nuclear volume to cell volume (including the yolk) varies from 1 : 89.5 to 1 : 34.8. In the different blastomeres of this egg there is no constant nuclear-plasmic ratio, or Kernplasma-Norm. However in different eggs corresponding blastomeres have the same Kernplasma-Relation, when measured at corresponding stages. The volumes of the protoplasm and of the nucleus show little variation in any given blastomere and the Kernplasma-Relation of each of the blastomeres named in table 3 is practically the same in all eggs. Since many of these blastomeres are peculiar in oöplasmic constitution and prospective significance it is not improbable that the peculiarities in their

TABLE 3

*Maximum nuclear size and cell size in the blastomeres of Crepidula plana; (measured just before nuclear membrane dissolves)*

STAGE BLASTOMERES	DIAMETER OF CELL	VOLUME OF CELL	DIAMETER OF PROTOPLASM INCLUDING NUCLEUS	DIAMETER OF NUCLEUS	VOLUME OF NUCLEUS	VOLUME OF PROTOPLASM LESS VOLUME OF NUCLEUS	KERN- PLASMA- RELATION	
	$\mu$	cubic $\mu$	$\mu$	$\mu$	cubic $\mu$	cubic $\mu$		
Before maturation.....	150	1,755,000	ca. 64*	42	32,409	97,131	1 : 3	
Before first cleavage.....	142	1,488,910	ca. 65*	$\sigma^2 24 = 34.5$ 930+	21,375	121,430	1 : 5.6	
AB, CD, before second cleavage.....	106	619,329	ca. 51*	24	7,238	61,741	1 : 8.5	
A, B, C, D, before third cleavage.....	82	286,712	ca. 44*	22	5,775	38,570	1 : 6.6	
1A-1D, before fourth cleavage.....	81	276,350	ca. 40	21	4,849	28,431	1 : 5.8	
1a-1d, before division.....				30	14	1,437	12,603	1 : 8.7
2A-2D, before fifth cleavage.....	80	266,240	ca. 36	18	3,055	21,196	1 : 7	
2a-2d, before division.....				36	15	1,767	22,484	1 : 12.7
1a <sup>1</sup> -1d <sup>1</sup> , before division.....				30	12	905	13,135	1 : 14.5
1a <sup>1</sup> -1d <sup>1</sup> , before division.....				15	7	180	1,387	1 : 8.8
3D, before sixth cleavage.....	76	228,288	ca. 30	16	2,145	11,895	1 : 5.5	
3A-3C, before sixth cleavage.....	76	228,288	ca. 22	16	2,145	3,439	1 : 1.6	
3a-3d, before division.....				33	14	1,437	19,350	1 : 13.3
2a <sup>1</sup> -2d <sup>1</sup> , before division.....				30	14	1,437	12,603	1 : 8.7
2a <sup>2</sup> -2d <sup>2</sup> , before division.....				30	14	1,437	12,603	1 : 8.7
4d, before seventh cleavage.....	38	28,533	ca. 22	11	897	4,878	1 : 7	
4A-4D, before seventh cleavage.....	60	112,320	ca. 20	18	3,055	1,134	1 : 0.37	
4a-4c, before seventh cleavage.....	42	32,409	ca. 14	12	905	532	1 : 0.58	

\* After yolk has been centrifuged out of egg. In normal eggs yolk and protoplasm are not well segregated at this stage.

Kernplasma-Relation may be the result of differentiations already present in the blastomeres.

In table 3 only maximum nuclear and cell dimensions are given for the different blastomeres. Results would undoubtedly differ greatly if the minimum nuclear and cell dimensions were taken instead of the maximum. Accordingly in table 4 the minimum nuclear and cell dimensions for the various blastomeres of *Crepidula plana* are given, together with the Kernplasma-Relation of each.

It is well known to cytologists that in cells undergoing regular division the minimum size of the nucleus is reached in the late anaphase, when the individual chromosomes have contracted to their smallest size and when they are most closely crowded together. A little earlier than this stage the chromatic plate is wider and the spaces between individual chromosomes greater; a little later the chromosomes begin to absorb achromatin and to swell up to form the chromosomal vesicles. At this stage of greatest nuclear contraction the chromosomal plate has approximately the form of a disk or short cylinder, and although the polar ends of the chromosomes are closer together than the equatorial ends, the disk being like a truncated cone, rather than a cylinder, we shall not greatly err if we treat this chromosomal disk as a short cylinder, rather than as a truncated cone. In table 4, in the column giving the dimensions of the chromosomal disk the first number is the diameter of the disk, the second its thickness.

Popoff ('08) has found in *Frontonia* that immediately after cell-division there is a diminution of the nucleus, which is then followed by a slow growth ('Funktionelles Wachstum' of Hertwig), and this by a much more rapid growth of the nucleus preceding division ('Teilungswachstum' of Hertwig). Both the functional growth and the divisional growth occur in the cleavage of *Crepidula*, but there is no diminution of the nucleus following division as in *Frontonia*. On the other hand, the minimum nuclear size is reached in the anaphase just before division of the cell body, as has been explained.

In the early telophase the chromosomal plate is drawn close to, and moulded over, the centrosome, and consequently the shape

of the chromosomal plate, its degree of curvature and width, dependent in part upon the size of the centrosome. I found in 1893 that in unequal cell division the centrosomes and asters become unequal before the cell division is finished, though in the earlier stages of mitosis the centrosomes and asters at the two poles of the spindle are equal in size; only after the cell division is finished do the daughter nuclei become unequal. The present work has confirmed these earlier conclusions and has shown in addition that the shape of the chromatic plate at the ends of the spindle is influenced by the size of the centrosomes, and hence by the equality or inequality of the division. If the centrosome is large the chromosomes form a slightly arched plate on its surface; if it is small the plate is highly arched. In the former case the plate remains relatively wide and the daughter nuclei when they are formed are disk-shaped; in the latter the plate and the daughter nuclei become more nearly spherical. Therefore, in comparing the sizes of chromatic plates it is necessary to measure them before this difference in shape appears, i.e., in the late anaphase. But even when all these precautions are taken the probable error in measuring objects of such small dimensions is considerable, but at least these measurements give the relative order of magnitude of the chromosomal disks in the different blastomeres.

The minimum cell dimensions occur in the early telophase, when the daughter cells first separate; at this stage the cells are nearly spherical in form and it is not difficult to calculate their volumes with substantial accuracy. While the minimum cell size does not occur at precisely the stage when the nuclei are smallest, it occurs so soon thereafter that it can make but little difference in the determination of the Kernplasma-Relation.

In short the Kernplasma-Relation, when plasma and nuclei are measured at their minimum sizes, varies in different blastomeres from 1:29 to 1:285.6. Except in the division of certain cells in the fourth and fifth cleavages ( $2A-2D$  and  $2a-2d$ ,  $2a^1-2d^1$  and  $2a^2-2d^2$ ) there is no appearance of a constant ratio between nucleus and plasma in these different blastomeres. In general the dimensions of the nuclear plates decrease with every cleav-

TABLE 4

*Minimum nuclear size and cell size in the blastomeres of Crepidula plana. (Nuclear plate measured in the late anaphase; cell diameter in early telophase)*

STAGE	BLASTOMERES	DIAMETER OF CELL	DIAMETER OF PHOTO-PLASM INCLUDING NUCLEUS	DIMENSIONS OF NUCLEAR PLATE	VOLUME OF NUCLEAR PLATE	VOLUME OF PHOTO-PLASM INCLUDING VOLUME OF NUCLEUS	KERNPLASMA-RELATION
		$\mu$	$\mu$	$\mu$	cubic $\mu$	cubic $\mu$	
2 cells	AB, CD.	105	ca. 46	9 x 3	190.5	30,504	1 : 160.
4 cells	A, B, C, D.	78	ca. 40	8 x 3	150.6	30,695	1 : 203.8
8 cells	1A-1D.	75	ca. 36	6 x 3	84.6	24,166	1 : 285.6
	1a-1d	27	27	6 x 3	84.6	10,235	1 : 120
12 cells	2A-2D.	72	ca. 30	6 x 3	84.6	13,955	1 : 165
	2a-2d	30	30	6 x 3	84.6	13,955	1 : 165
16 cells	1a <sup>1</sup> -1d <sup>1</sup>	30	30	5 x 3	58.8	13,955	1 : 237
	1a <sup>2</sup> -1d <sup>2</sup>	15	15	5 x 3	58.8	1,708	1 : 29
20 cells	3A-3D.	72	ca. 15	4 x 3	37.5	1,729	1 : 46
	3a-3d	25	25	4 x 3	37.5	8,087.5	1 : 215
24 cells	2a <sup>1</sup> -2d <sup>1</sup>	24	24	4 x 3	37.5	7,200	1 : 195
25 cells	4D.	60		5 x 3	58.8		
	4d	30		5 x 3	58.8		
29 cells	2a <sup>2</sup> -2d <sup>2</sup>	24	24	4 x 3	37.5	7,200	1 : 195
	1a <sup>1-1</sup> -1d <sup>1-1</sup>	15	15	4 x 3	37.5	1,729.5	1 : 46
32 cells	1a <sup>1-2</sup> -1d <sup>1-2</sup>	24	24	4 x 3	37.5	7,200	1 : 195
	4A-4C.			4 x 3	37.5		
	4a-4c.			4 x 3	37.5		

age, while all the cells of the same generation have nuclear plates of about the same size, though their protoplasmic volumes vary widely.

There is a great difference between the maximum and minimum volumes of the same nucleus, ranging from 1:27 to 1:38, while there is a relatively slight difference between the maximum and minimum sizes of the plasma and accordingly the Kernplasma-Relation of any blastomeres varies continuously from the stage of minimum nuclear volume to that of maximum nuclear volume: Hertwig ('08) has chosen as the stage showing the Kernplasma-Norm, "das Verhalten der jugendlichen Zelle, welche eben aus der Teilung hervorgegangen ist und nun anfängt sich

von neuem zu ernähren, um abermals heranzuwachsen und sich zu teilen." I have tried to make measurements at the stage described by Hertwig, but find that in these segmenting eggs it is too ill defined to be safely used. Between successive divisions the nuclei are growing continuously and rapidly and there is no clearly marked pause in the nuclear growth; accordingly slight differences in the stages chosen for measurement show relatively large differences in the sizes of the nuclei.

In order to take a stage intermediate between the two extremes of nuclear size, and in which the nuclei may be regarded as having reached a normal functional condition, not related primarily to the preceding or succeeding division, I have chosen that stage when the nuclei first become regularly spherical in shape. For a considerable part of the resting period the nuclei are elongated at right angles to the previous spindle axis, and in the plane of the chromatic plate of the previous anaphase; during the latter part of the resting period they grow very rapidly in preparation for the succeeding division ('Kernteilungswachstum' of Hertwig). The stage when the nuclei first become spherical lies somewhere between these two phases and may therefore be considered to represent the mean nuclear size. However this stage is not so precisely defined as are the stages of maximum and minimum nuclear size, and therefore the nuclear dimensions are likely to be more variable.

It is obviously more difficult to determine the volume of the cleavage cells during the resting period, when they are pressed into irregular and polygonal forms, than during mitosis when they approach a spherical shape. However the approximate accuracy of the cell dimensions recorded in table 5 may be judged by comparing them with the maximum and minimum cell dimensions, given in tables 3 and 4.

The Kernplasma-Relation varies about as much for mean dimensions of the nucleus and cell as for maximum ones, though not as much as for minimum dimensions. In yolk-containing cells it varies from 1:1.1 to 1:27.5 and in purely protoplasmic cells from 1:7 to 1:35.7.



TABLE 5

Mean nuclear size and cell size in the blastomeres of *Crepidula plana*. (Measured when nuclei first become spherical after division)

STAGE	BLASTOMERES	DIAMETER OF CELL	DIAMETER OF PROTOPLASM INCLUDING NUCLEUS	DIAMETER OF NUCLEUS	VOLUME OF NUCLEUS	VOLUME OF PROTOPLASM LESS VOLUME OF NUCLEUS	MEMBRANE-RELATION
		$\mu$	$\mu$	$\mu$	cubic $\mu$	cubic $\mu$	
Before cleavage		136	ca. 60	$\left\{ \begin{array}{l} \text{♀ } 18 \\ \text{♂ } 12 \end{array} \right.$	3,960	109,137	1 : 27.5
2 cells	AB, CD	105	ca. 44	18	3,055	41,240	1 : 13.5
4 cells	A, B, C, D	78	ca. 40	16	2,145	31,135	1 : 14.5
8 cells	$\left\{ \begin{array}{l} 1A-1D \\ 1a-1d \end{array} \right.$	75	ca. 36	15	1,767	22,484	1 : 12.7
	$\left\{ \begin{array}{l} 2A-2D \\ 2a-2d \end{array} \right.$	30	30	12	905	13,135	1 : 14.5
16 cells	$\left\{ \begin{array}{l} 2A-2D \\ 2a-2d \end{array} \right.$	72	ca. 36	15	1,767	22,484	1 : 12.7
	$\left\{ \begin{array}{l} 1a^1-1d^1 \\ 1a^2-1d^2 \end{array} \right.$	36	36	12	905	23,346	1 : 25.6
	$\left\{ \begin{array}{l} 3A-3D \\ 3a-3d \end{array} \right.$	30	30	9	382	13,658	1 : 35.7
20 cells	$\left\{ \begin{array}{l} 1a^1-1d^1 \\ 1a^2-1d^2 \end{array} \right.$	15	15	6	113	1,654	1 : 14.6
	$\left\{ \begin{array}{l} 3A-3D \\ 3a-3d \end{array} \right.$	72	ca. 18	14	1,437	1,618	1 : 1.1
	$\left\{ \begin{array}{l} 2a^1-2d^1 \\ 2a^2-2d^2 \end{array} \right.$	30	30	12	905	13,135	1 : 14.5
24 cells	$\left\{ \begin{array}{l} 2a^1-2d^1 \\ 2a^2-2d^2 \end{array} \right.$	27	27	12	905	9,330	1 : 10.3
	$\left\{ \begin{array}{l} 4D \\ 4d \end{array} \right.$	27	27	12	905	9,330	1 : 10.3
25 cells	$\left\{ \begin{array}{l} 4D \\ 4d \end{array} \right.$	60					
	$\left\{ \begin{array}{l} 1a^{1,1}-1d^{1,1} \\ 1a^{1,2}-1d^{1,2} \end{array} \right.$	34		9	382		
29 cells	$\left\{ \begin{array}{l} 1a^{1,1}-1d^{1,1} \\ 1a^{1,2}-1d^{1,2} \end{array} \right.$	15	15	6	113	1,654	1 : 14.6
	$\left\{ \begin{array}{l} 4A-4C \\ 4a-4c \end{array} \right.$	24	24	12	905	6,333	1 : 7
32 cells	$\left\{ \begin{array}{l} 4A-4C \\ 4a-4c \end{array} \right.$			15	1,767		
				9	382		

Incidentally the interesting fact appears that nuclei in large cells do not become spherical until they have reached a larger size than their sister nuclei in small cells; for example in the micromeres *1a-1d* and *2a-2d* the nuclei become spherical when they are about  $12\mu$  in diameter, in the macromeres *1A-1D* and *2A-2D* their sister nuclei are about  $15\mu$  in diameter before they become spherical. Since the same amount of chromatin goes into each of the sister nuclei, the difference in the size of the nuclei when they first become spherical must be found in some other factor. It seems probable that it is due to the shape of the chromosomal disk, which remains flattened, or slightly arched, in large cells

and is highly arched in small ones, owing to the greater or smaller size of the centrosomes, as explained on p. 19. The flattened chromosomal plate gives rise to a disk-shaped nucleus, which only later becomes spherical, whereas the highly arched plate sooner gives rise to a spherical nucleus.

2. *Cell-size and nuclear size in the cleavage of Fulgur carica.* The eggs of *Fulgur carica* are the largest gasteropod eggs of which I know, while the eggs of *Crepidula plana* are among the smallest. It will be instructive therefore to compare the Kernplasma-Relation in these two cases. Table 6 gives the mean nuclear size and cell size of the blastomeres of *Fulgur*. The eggs measured were fixed, stained and mounted entire, as in the case of *Crepidula plana*. Owing to the great size of these eggs it was necessary

TABLE 6  
Mean nuclear size and cell size in the blastomeres of *Fulgur carica*

	DIAMETER OF CELL	DIAMETER OF PROTOPLASM	DIAMETER OF NUCLEUS	VOLUME OF NUCLEUS	VOLUME OF PROTOPLASM LESS VOL- UME OF NUCLEUS	KERNPLASMA- RELATION
	$\mu$	$\mu$	$\mu$	cubic $\mu$	cubic $\mu$	
<i>Macromeres</i>						
AB, CD, before second cleavage.....	1200	200	40	33,280	4,126,720	1 : 124
A, B, C, D, before third cleavage.....	800	160	32	17,040	2,112,880	1 : 124
1A-1D, before fourth cleav- age.....	800	160	32	17,040	2,112,880	1 : 124
2A-2D, before fifth cleav- age.....	800	160	32	17,040	2,112,880	1 : 124
3D, before sixth cleavage.	800	160	32	17,040	2,112,880	1 : 124
3A-3C, before sixth cleav- age.....	800	160	48	57,508	2,062,412	1 : 35.8
4A-4D, before seventh cleavage.....	768	160	96	460,063	1,669,857	1 : 3.6
<i>Micromeres</i>						
1a-1d.....		80	16	2,145	264,095	1 : 127.7
2a-2d.....		80	16	2,145	264,095	1 : 127.7
3a-3d.....		80	16	2,145	264,095	1 : 127.7
1a <sup>1</sup> -1d <sup>1</sup> .....		80	16	2,145	264,095	1 : 127.7
1a <sup>2</sup> -1d <sup>2</sup> .....		40	8	266	33,014	1 : 124

to make the measurements under a relatively low power, the 8 mm. apochromat objective and the 1/1 micrometer eyepiece of Zeiss. Owing to the relatively low magnification the probable error is greater than in the measurements of the eggs of *C. plana*.

With the exception of the cells *3A-3C* and *4A-4D* the Kernplasma-Relation is in this case practically constant, varying only from 1 : 124 to 1 : 127. In view of the fact that I can find no constant Kernplasma-Relation in the blastomeres of *Crepidula* this result in the case of *Fulgur* is unexpected. I am sure that my measurements in the case of *Fulgur* are not so accurate as in *Crepidula*, the number of eggs measured being relatively small and the magnification used low, so that each interval of the scale stood for  $8\mu$ . The uniformity in the measurements of the different blastomeres and nuclei of *Fulgur* may be due in part to this fact; on the other hand this would only account for the lack of minor variations and would not explain the general uniformity. There is no doubt that the micromeres of *Fulgur* are more uniform in size, than those of *Crepidula*; also the whole cleavage process is very much slower, and (with the exception of the macromeres *3A-3C* and *4A-4D*) the divisions are more nearly synchronous in the different cells than in *Crepidula*. It seems probable that these two facts are connected with the more uniform Kernplasma-Relation of the different blastomeres of *Fulgur*.

This conclusion is rendered still more probable by a consideration of the two generations of cells in which there is a wide departure from the usual Kernplasma-Relation, viz. *3A-3C* and *4A-4D*. In these cases, as in the same cells in *Crepidula* the resting stage is particularly long, lasting in the case of *4A-4D* until all the organs of the embryo are outlined and more than one thousand cells are present; consequently the nuclei grow to an enormous size so that the Kernplasma-Relation falls in one case to 1 : 35.8 and in the other to 1 : 3.6. In the corresponding cells in *Crepidula* the ratio is 1 : 1.6 and 1 : 0.37; the volume of the nucleus in the last named case being about three times that of the plasma. The Kernplasma-Relation of the cells *4a-4c* is 1 : 0.58 in *Crepidula*, the nuclei being about twice as voluminous as the plasma; in the corresponding cells of *Fulgur* this ratio cannot be

readily determined since the nuclei undergo several divisions, though the cell body does not divide.

From these measurements it may be concluded that when cell division takes place at regular intervals the Kernplasma-Relation is fairly constant; when it takes place at irregular intervals this ratio is variable. The longer the resting period the larger the nucleus becomes, and in extremely long resting periods the greater part of the plasma may be taken up into the nucleus.

These observations are in full agreement with experiments on the eggs of *Crepidula* which will be described later. They are not antagonistic to Boveri's conclusions as to the correlation between chromosome number and nuclear size; on the other hand my own experiments show that the size of the nucleus is dependent, in part, upon the number of chromosomes which enter into its formation. But in normal cells all of which contain the same number of chromosomes differences in nuclear size must be due to some other factor.

The results of my measurements do not indicate that the Kernplasma-Relation of Hertwig is either a constant or self regulating ratio in the blastomeres of these eggs; on the other hand it appears to be a result rather than a cause of the rate of cell division, and consequently it is a variable rather than a constant factor. Furthermore the size of the nucleus, in these eggs, is dependent upon at least three factors: (1) The initial quantity of chromatin (number of chromosomes) which enter into the formation of the nucleus (Boveri). (2) The volume of the protoplasm in which the nucleus lies. (3) The length of the resting period.

### *III. Cell size and nuclear size in adult tissue cells*

It is generally believed that embryonic cells differ greatly from adult tissue cells in their "Kernplasma-Relation." In a series of thoughtful and suggestive works Minot ('90, '95, '08) has maintained that differentiation, senescence and finally death are the accompaniments, if not the results, of an increase of protoplasm as compared with nucleus. It is well known that embryonic cells of plants are more purely protoplasmic than adult cells,

which are frequently filled with vacuoles and sap so that the size of the cell gives no true idea of the volume of the cytoplasm. Among animals adult tissue cells often become filled with the products of differentiation or metabolism, such as fibers, granules, secretions, oil, etc., which greatly increase the cell dimensions. It is evidently a difficult if not impossible task to determine the quantity of real protoplasm in such cells and thus to discover the true "Kernplasma-Relation." However in certain less highly differentiated cells, especially in epithelial and glandular tissue, the true Kernplasma-Relation may be established with a fair degree of accuracy.

Unquestionably the physiological state of a cell has much to do with its nuclear-plasmic ratio. Hodge ('92) found the nuclei of nerve cells shrunken after extreme stimulation, and it has been long known that the same is true of gland cells. In *Crepidula* the liver cells, when active, are filled with secretion and are among the largest in the body, but when the secretion has been discharged and they have returned to an inactive condition, the cell body is much smaller and the nucleus larger.

I have measured the cells and nuclei of a number of tissues of *Crepidula plana*, derived from the three germ layers, and the results are given in table 7. Since these cells vary in shape to a great extent, and in order to facilitate comparison of cell diameter and nuclear diameter, cells were chosen for measurement which were as nearly as possible spherical or cubical in shape. In all elongated cells the long axis and one cross axis were measured and it was assumed that the other cross axis was of the same dimensions as the one observed.

It is evident that in these tissue cells of *Crepidula plana* there is no marked increase of protoplasm over nucleus as compared with the blastomeres of the same species; throughout the cleavage, with the exception of the cells 3A-3D and 4A-4D, the average Kernplasma-Relation for nuclei and cells of mean size is about 1 : 15, for nuclei and cells of maximum size about 1 : 6; the average ratio in adult tissue cells, which are not filled with metabolic products, is about 1 : 10.5. In the case of the ganglion cells the nuclei are relatively and absolutely larger than in the other tissues,

TABLE 7

*Cell size and nuclear size in tissue cells of sexually mature individuals of Crepidula plana*

TISSUE CELLS	DIMENSIONS OF CELL	DIAMETER OF NUCLEUS	VOLUME OF NUCLEUS	VOLUME OF CELL, LESS VOLUME OF NUCLEUS	KERN-PLASMA RELATION
	$\mu$	$\mu$	cubic $\mu$	cubic $\mu$	
Intestinal epithelium....	11 x 11 x 12	6	113	1,339	1 : 11.8
Gastric epithelium.....	10 x 10 x 36	8	68	3,332	1 : 12.4
Liver duct epithelium....	10 x 10 x 18	6	113	1,628	1 : 14.4
Liver cells (filled with secretion products).....	15 x 15 x 45	6*	113	10,012	1 : 88.6
Liver cells (without secretion products).....	14 x 14 x 30	9	382	5,498	1 : 14.4
Kidney cells (containing secretion products)....	15 x 15 x 15	6	113	3,262	1 : 28.8
Ectodermal epithelium (near anus).....	5 x 5 x 15	4	33	342	1 : 10.3
Gill chamber epithelium..	6 x 6 x 12	4	33	405	1 : 12.2
Gill filament epithelium..	7 x 7 x 9	4	33	408	1 : 12.3
Epithelium from foot....	6 x 6 x 15	5	65.4	474.6	1 : 7.1
Ganglion cell (large).....	17 x 17 x 23	12	905	5,724	1 : 6.3
Ganglion cell (large).....	10 x 10 x 20	9	382	1,618	1 : 4.2
Oöcytes I (before yolk formation).....	12 $\frac{1}{2}$	7	180	836	1 : 4.6
Oöcytes I (before yolk formation).....	11 $\frac{1}{2}$	7	180	791	1 : 3.4
Oöcytes I (before yolk formation).....	10	6	113	407	1 : 3.6
Oöcytes I (before yolk formation).....	8	5	65.4	203	1 : 3.1
Oöcytes I (before yolk formation).....	6 $\frac{1}{2}$	4	33	111	1 : 3.3

\*Nucleus shrunken and very irregular in shape.

the Kernplasma-Relation being about 1 : 5; however in this case the nerve fiber is not added to the cell body and this would doubtless greatly increase the volume of the plasma. Muscle cells in *Crepidula* are long, slender and crooked and I have found it impracticable to estimate their volumes with any degree of accuracy. Doubtless the plasma, including the contractile substance, is here relatively much more abundant than in embryonic or epithelial cells. In the epithelial and gland cells of adult

*Crepidula* the embryonic ratio of nucleus to plasma is maintained with little change. In all the oöcytes up to the time that yolk formation begins the nuclei are relatively large, the ratio of nucleus to plasma being about 1 : 3.6, and in the younger and smaller oöcytes the nuclei are relatively larger than in the older and larger ones.

Eycleshymer ('04) found that the volume of the plasma in the striated muscle cells of *Necturus* increased about ten times as much as the nuclear volume, during development from the 8 mm. embryo to the adult condition. There is, therefore, in these later stages a notable shifting of the Kernplasma-Relation in favor of the plasma. It is probable however that the contractile substance which makes up the larger part of the muscle cell, does not contribute to the growth of the nucleus as does the protoplasm of embryonic cells—that so far as the growth of the nucleus is concerned it acts as does yolk, oil, membranes, fibers and other products of metabolism and differentiation. If only the sarcoplasm of the muscle cell and not its contractile substance is able to contribute to the growth of the nucleus, the small volume of the nuclei as compared with the entire cell would find a ready explanation. There can be no doubt that the plasma is the chief seat of differentiation, as Minot has emphasized, and that highly differentiated cells, such as muscle, nerve, and some kinds of connective tissue, have a larger amount of plasma and its products, relative to the nucleus, than have embryonic cells. In the case of fiber cells, fat cells, and probably muscle cells, the cell body becomes filled with the products of differentiation and metabolism, which like the yolk in egg cells, or the secretion products in liver cells cannot enter the nucleus and consequently do not influence its size. In such tissue cells the cell body is relatively much greater as compared with the nucleus, than in purely protoplasmic cells, but I have been unable to find any evidence that the ratio of protoplasm (using this term in its usual sense) to the nucleus is greater in tissue cells of *Crepidula* than in the blastomeres.

*IV. The inciting causes of cell division*

The relative sizes of cells and of nuclei are dependent, in part, upon the rate of cell division. Cells which divide infrequently are larger, other things being equal, than those which divide often. The turret cells ( $1a^2-1d^2$ ) of *Crepidula* are the smallest cells in the entire embryo at the time of their formation (figs. 3, 4); however they divide but twice during the whole of the cleavage period, and consequently they grow to be very large; whereas each of the apical cells from which they were derived, gives rise during the cleavage period to twelve cells the combined volume of which is not much greater than that of one full-grown turret cell. Evidently the factors which bring on or delay cell-division have much to do, indirectly, with the sizes of cells and nuclei.

Strasburger ('93) supposed that cell division occurred when the ratio of the cell body to the nucleus increased beyond a certain point, which might be regarded as marking the limit of the 'working sphere of the nucleus;' with the division of the cell the normal ratio was once more restored.

Boveri ('04) sought to find the inciting cause of cell division in the chromosomes. He believed that the chromosomes divide when they have reached a size double that which they had at the close of the preceding division. At the same time he showed that the rhythm of the division of the centrosomes may be independent of that of the chromosomes and that division of the cell depends upon the centrosomal rhythm rather than upon the chromosomal rhythm.

That there is a rhythm of division for chromosomes and centrosomes seems to be well established by Boveri's work, but this rhythm in the case of the chromosomes is not determined by the time when they have grown to double their size at the close of the preceding division. Marcus ('06) and Erdmann ('08) have shown that the chromosome size throughout the cleavage of *Strongylocentrotus* is a constantly decreasing one. Baltzer ('08) admits that the chromosomes do not double in size at each cycle of division; he does not find any great diminution in chromosome size up to the 16-cell stage, though the chromosomes in the blastula



stage are undoubtedly smaller than those of early cleavage stages. In *Crepidula* the chromosomal plate decreases in size in successive cleavages, though by no means uniformly; but at no time during the cleavage period do the chromosomes grow to their original size at the beginning of the cleavage. Boveri's view, therefore, finds no support in the cell-divisions of the cleavage period.

R. Hertwig ('03, '08) finds the inciting cause of division in a 'Kernplasma-Spannung,' due to the unequal growth of nucleus and plasma:

Die Kernplasma-Relation muss eine Verschiebung erfahren zuungunsten des Kernes, es muss sich eine Kernplasma-Spannung entwickeln, welche allmählich zunimmt, bis schliesslich ein Grad erreicht wird, den ich früher Kernplasma-Spannung in engeren Sinne genannt habe. In dieser Spannung erblicke ich die Ursache der Teilung. Ich nehme an, dass, wenn ein Höhepunkt der Kernplasma-Spannung erreicht wird, der Kern die Fähigkeit gewinnt, auf Kosten des Protoplasma zu wachsen, und das die hierbei sich vollziehenden Stoffumlagerungen zur Teilung der Zelle führen. Zum funktionellen Wachstum gesellt sich das Teilungswachstum des Kernes, um die Kernplasma-Norm wiederherzustellen." (p. 20)

Relative Zunahme der Kernsubstanz, gleichgültig, ob dieselbe durch Vergrösserung des Kernes bei gleichbleibender Protoplasamenge oder Verringerung des Protoplasma bei gleichbleibender Kerngrösse herbeigeführt wird, müsste eine Verlangsamung der Teilung und im ersten Fall eine Steigerung der Teilgrösse zur Folge haben; umgekehrt müsste relative Abnahme der Kernmasse den Eintritt der Kernteilung beschleunigen, die Teilgrösse herabsetzen (p. 23).

Hertwig holds that his own work on Infusoria, and that of Gerassimoff on *Spirogyra*, show that an increase of nuclear mass leads to a slowing of divisions and an increase of the division size of the cell; and that the process of the segmentation of the animal egg shows that a great reduction of nuclear mass leads to a high degree of divisional activity. He says that many external and internal conditions influence the Kernplasma-Relation and he expresses the hope that his theory may not be cast aside because here and there a fact may be found which cannot be brought under it, without further consideration.

As we have seen the Kernplasma-Relation varies widely in certain blastomeres of *Crepidula* and *Fulgur*. In these cases wide departures from the Kernplasma-Norm have not brought

on cell division, and if Kernplasma-Spannung is a cause of cell-division it must be a minor factor in this case. It seems to me probable from my observations and experiments on segmenting eggs, that the Kernplasma-Relation in these blastomeres is a result rather than a cause of the rhythm of cell division, and that the factors which bring on cell division are to be found in some intrinsic condition in the nucleus or centrosome, rather than in the maintenance of a constant ratio of nuclear volume to cell volume. Support is lent to this view by the phenomena of oögenesis, for we have in the germinal vesicle the largest nucleus in the entire life cycle, following upon the longest resting period, while the second maturation division follows immediately upon the first, usually before a resting nucleus is formed. The long delay in the appearance of the first maturation division, as well as the short period intervening between the first and second maturation divisions, must both be attributed, as it seems to me, to intrinsic conditions in the cell, other than 'Kernplasma-Spannung.'

In the cleavage of the egg the rate of division seems to depend, in part, on the quantity of protoplasm present. As long as a considerable quantity of plasma is present in the blastomeres the rate of division is rhythmical, but when the macromeres have given off almost all the plasma in the formation of the three quartets of ectomeres, a long resting period follows. The first of these macromeres to divide, giving rise to the fourth quartet, is the one with the largest amount of plasma, viz., 3*D*, while the cells 3*A*-3*C* normally divide much later. However if, by centrifuging at the right stage, 3*C* is caused to contain more plasma than usual it may divide at the same time as 3*D*, as shown in fig. 37. The cells 4*A*-4*D*, in which the resting period is particularly long, contain very little plasma, and this appears to be absorbed by the nucleus almost as fast as it is formed. The micromere 1*d* is slightly smaller than its fellows, 1*a*-1*c*, and it divides later than the latter. The 'turret' cells, 1*a*<sup>2</sup>-1*d*<sup>2</sup>, are the smallest cells in the egg, when they are formed, and they have the longest resting period.

In spite of this evidence that the quantity of protoplasm has to do with the rate of division, there is other conflicting evidence which is hard to harmonise with it; thus, these same 'turret' cells,

which are at first so small and have so long a resting period, become much larger than adjoining cells before they divide. R. Lillie ('10) maintains that "the primary change in the initiation of cell division and development is an increase in the permeability of the plasma membrane." It is well known that the general surface tension of the cell increases during mitosis, and I have found that the tension of the cell membrane is locally reduced at the two poles of the cell before and during division (see Conklin, '02, p. 94; also this paper, p. 82). It is quite possible that this polar reduction in surface tension before mitosis begins may have something to do with initiating division.

On the whole it seems probable that the time of cell division is dependent upon the coincidence of several more or less independent factors. Boveri has shown that the division phases in nucleus and centrosome may be more or less independent of each other, though complete cell division depends upon the coincidence of the two. To these factors may, perhaps, be added the quantity of protoplasm, and thus indirectly the 'Kernplasma-Relation' and perhaps also increased permeability of the cell membrane, and a local reduction of surface tension at the poles of the cell. Gurwitsch ('08) maintains that the blastomeres are ready for division at all times, and that only 'Kernplasma-Koinzidenz' or 'Zustands-Koinzidenz,' is necessary to start division. He suggests that a coincidence of polarity of nucleus and plasma may be necessary, and he concludes from the apparently accidental occurrence of divisions in different parts of an egg or embryo, that several independently variable factors may be concerned, the coincidence of which is necessary to bring on cell division. The latter part of this conclusion seems to me to be justified by the facts which I have presented.

#### V. *Growth of protoplasm during cleavage*

It is well known that the egg as a whole does not increase in volume until after the cleavage period. Indeed Godlewski ('08) finds that there is in *Echinus* and in *Strongylocentrotus*, no change in the quantity of plasma at the 64-cell stage, as compared with the

unsegmented egg; however, in the blastula there is an actual loss, the total volume of plasma being about one-third less than in the unsegmented egg; during this period the nuclear material has increased in volume at the expense of the plasma. Whether the plasma actually increases during cleavage at the expense of the yolk has not been determined, so far as I am aware, in any case. By means of the centrifuge it is possible to throw the yolk out of the egg before cleavage and during the early cleavage stages, leaving the plasma which can then be readily measured. In later cleavage stages I have not been able to throw the yolk out of the small blastomeres by means of the centrifuge; but on the other hand the protoplasm and yolk are normally segregated in these stages so that it is possible to determine the approximate dimensions of both without having recourse to the centrifuge. The following table gives the total maximum volumes of all the nuclei, protoplasm and yolk in the eggs of *Crepidula plana* at various cleavage stages. Following Popoff ('08), I have determined the coefficients of growth of the nucleus and of the protoplasm for each stage; these coefficients are obtained by dividing the volume of a later stage by that of an earlier one, and they represent the growth in 'times,' or multiples of the initial quantity. In the first half of each column of coefficients the earlier stage is the one before maturation, while in the second half of each column it is the one before the first cleavage. The coefficient of growth of

TABLE 8  
Total maximum volumes of nuclei, protoplasm and yolk in the eggs and cleavage stages of *Crepidula plana*

STAGE	VOLUME OF NUCLEI	VOLUME OF PROTOPLASM	VOLUME OF YOLK	TOTAL VOLUME OF EGG	COEFFICI- ENTS OF NUCLEAR GROWTH	COEFFICIENTS OF PROTOPLASMIC GROWTH	TOTAL KERN- PLASMA- RELATION
	<i>cubic <math>\mu</math></i>	<i>cubic <math>\mu</math></i>	<i>cubic <math>\mu</math></i>	<i>cubic <math>\mu</math></i>			
Before ma- turation.....	52,409	97,131	1,625,460	1,755,000	1.0	1.0	1:3
Before first cleavage.....	21,375	121,430	1,346,105	1,488,910	0.65	1.0	1:5.6
2 cells.....	14,476	123,482	1,100,700	1,238,858	0.45	0.67	1:8.5
4 cells.....	23,100	154,280	969,468	1,146,848	0.71	1.03	1:6.6
8 cells.....	25,144	184,136	972,320	1,161,900	0.77	1.17	1:6.6
16 cells.....	23,628	233,608	980,156	1,237,392	0.72	1.10	1:9.8
24 cells.....	30,164	258,897	890,727	1,179,788	0.92	1.41	1:8.6
		(231,000)		(1,151,891)		(2.35)	(1:7.7)

any stage, less the coefficient of the initial stage, viz. unity, gives the percentage of growth of that stage, as compared with the initial stage.

Since the ectoderm at the 24-cell stage is a plate of purely protoplasmic cells, nearly square, about  $80\mu$  on each side and  $36\mu$  thick its volume is about 230,400 cubic  $\mu$ ; subtracting the volumes of the nuclei of the plate, 21,584 cubic  $\mu$ , leaves 208,816 cubic  $\mu$  as the volume of the cytoplasm<sup>1</sup> of the ectodermal plate. Adding to this the volume of the protoplasm in the macromeres 3A-3D, viz. 22,185 cubic  $\mu$ , we have as the total volume of the protoplasm at the 24-cell stage 231,000 cubic  $\mu$ . This figure is 27,897 cubic  $\mu$  less than the volume of protoplasm at the 24-cell stage given in the table, which was calculated from the dimensions of each individual cell, rather than from those of the entire ectodermal plate. It is highly probable that the lower figure is nearer correct than the higher one, since minor errors in the measurements of individual cells are greatly magnified in determining the total volumes of these cells. The same remark applies to the total volume of protoplasm in the 16-cell stage, which is probably actually less than the volume given in the table; and if the total volume of the protoplasm is less than the amount given in the table the total volume of the yolk in these stages is of course increased correspondingly.

But assuming that the smaller number (in brackets) represents the actual volume of the protoplasm in the 24-cell stage of *Crepidula plana* we must admit that there has been a great growth in the plasma at the expense of the yolk during the cleavage. The coefficient of protoplasmic growth (i.e., the volume of protoplasm of any stage divided by the volume of protoplasm of the stage just before maturation) is given in the next to the last column of the table; and a glance at this shows that the protoplasm at the 24-cell stage is at least  $2\frac{2}{3}$  times as voluminous as in the maturation stage, while the yolk is correspondingly less voluminous. The volume of the entire egg, also, is considerably less in the 24-cell stage than at the beginning of development. Indeed there has been a gradual decrease in the volume of the entire egg during

<sup>1</sup> The words 'cytoplasm' and 'protoplasm' are used synonymously throughout this paper.

the early cleavages. These results show a general agreement with those of Godlewski.

The growth of plasma at the expense of yolk during the maturation and the cleavage period, was shown to occur in my studies of the effects of centrifugal force on the eggs of *Lymnaea* and *Physa* (Conklin, '10). In the living eggs of these animals the substances may be stratified by centrifugal force into a gray (light) zone, a clear (middle) zone and a yellow (heavy) zone; the gray and clear zones constitute what I have here regarded as protoplasm, while the yellow zone is in large part composed of yolk. "Before the first maturation the yellow substance composes at least one-half of the entire egg; just before the first cleavage it composes only about one-eighth of the egg. The clear and gray substances, which together constitute about one-half of the egg in the earlier period, form seven-eighths of the egg in the later period," (p. 436).

In the normal eggs of *Lymnaea* and *Physa*, which have not been centrifuged, the clear and yellow substances are easily recognizable, and the stages in the transformation of the latter into the former have been studied in the paper mentioned, from which the following summary is quoted:

In the course of development, from the maturation of the egg to the gastrulation, the relative quantities of clear (plasma) and yellow substance (yolk) are reversed. At the beginning the clear substance is small in quantity, and is chiefly visible in the germinal vesicle (though experiments show that some of it is distributed through the yellow substance) and at this stage the entire cell body is yellow in color. With the establishment of the germinal layers the yellow substance is limited to the few cells constituting the endoderm and mesoderm, while all the rest of the embryo, by far the larger part, is composed of clear substance. This change in the relative quantities of these two substances is due in part to their separation and segregation during the course of development, but in much greater part to the transformation of yellow substance (yolk) into the clear (plasma). It is a phenomenon of general occurrence among many animals that the clear protoplasm of the egg is very small in quantity before the dissolution of the germinal vesicle and that it gradually increases in quantity after that stage. This is doubtless due in large part to the dissolving of yolk and its conversion into clear protoplasm, and it is a significant fact that this process takes place most rapidly after the breaking down of the wall of the germinal vesicle and the escape of a large part of the nuclear contents into the cell body (p. 423).

There are no eggs wholly without yolk and probably in all of them plasma is formed at the expense of yolk during the cleavage period. This probability is of great significance, for all studies which have had to do with the relative quantities of protoplasmic and nuclear materials during these early stages of development have dealt only with the entire cell contents without attempting to determine what part of this is plasma. In many cases, the great disproportion between cell volume and nuclear volume at the beginning of development is due to the fact that a large part of the cell volume is made up of yolk; if the volume of the plasma only is compared with that of the nucleus it is found that the relative quantity of plasma is actually less at the beginning of development, than in the later cleavages, with the single exception of those blastomeres which have unusually long resting periods. In *Crepidula* there is no excess of plasma over nuclear material in the early stages, in comparison with the later ones, as Minot and others have assumed, and the process of cleavage is not in this case a method of restoring the Kernplasma-Norm, or of rejuvenating senile cells, by an enormous increase of nuclear material as compared with the plasma. As a matter of fact the plasma increases almost as rapidly as the nuclear material during the cleavage of this egg, and even adult tissue cells have a Kernplasma-Relation but little different from that of the blastomeres, (see p. 25).

#### VI. *Rate of nuclear growth during cleavage*

It is well known that during cleavage there is usually no increase in the volume of the egg, but it is generally held that the increase in the nuclear substance is very great. In his book on "Age, Growth and Death" Minot ('08) says: "The nuclei multiply (in cleavage); they multiply at the expense of the protoplasm. They take food from the material which is stored up in the ovum, nourish themselves by it, grow and multiply until they become the dominant part in the structure" (p. 166). He suggests that this nuclear increase during cleavage is a process of rejuvenation, though he admits that the relative increase of nu-

clear material as compared with protoplasmic may be prolonged beyond the period of segmentation (p. 167). But although he emphasizes the growth of the nuclear material as a whole during the cleavage, he specifically recognizes the fact that there is a rapid reduction in the size of individual nuclei in the early stages (pp. 174, 179). Hertwig ('03) also has emphasized this great growth of the nuclear material during the early stages of development. He says (p. 116):

There is an enormous disproportion of nucleus and protoplasm at the beginning of cleavage, and this disproportion is gradually equalized by the transformation of cell substance into nuclear substance. The manner of this may be imagined by supposing that resting protoplasm contains chromatin and achromatic material and that at every cell division it is analysed into these constituents serving for the growth of the nucleus.

Loeb ('09) also has called attention to the doubling of nuclei at each division, with the consequent increase of nuclear material in a geometric ratio, and the resemblance which this bears to autokatalytic reactions.

The great increase in the nuclear substances during cleavage has been commented upon by many writers, and the references cited have been chosen rather because of the theories which have been based upon this phenomenon than because they represent an unusual opinion as to the phenomenon itself. At the time when the following computations of the rate of nuclear growth during cleavage were made, I was unaware that anyone had made computations of a similar sort. Since my material afforded an unusually good opportunity for making such computations, I carefully measured the diameters of the germinal vesicle, of the egg and sperm nuclei, of all the nuclei up to the 24-cell stage, of those of the 42-cell stage, and of the 70-cell stage,—every nucleus being measured at its maximum size, so far as possible,—with the results given in the following tables. These results have been as surprising to me as they are likely to be to any of my readers.

After this work was completed I became acquainted with the work of Godlewski ('08) and Frl. Erdmann ('08) on the sizes of nuclei and of individual chromosomes of the blastomeres of



Echinus and Strongylocentrotus. Godlewsky found that from the 1-cell to the 64-cell stage the nuclear substance grows nearly in geometric ratio; from the 64-cell stage to the blastula, with about 1256 cells, there is little increase in the nuclear substance, but since he supposes that the number and size of the chromosomes in the later stages remain the same as in the earlier ones, the nuclei must become richer in chromatin in the later stages. He finds that the volume of the plasma in the blastula stage is about one-third less than in the unsegmented egg and he considers that a large part of this lost plasma has been converted into chromatin. Erdmann ('08) has made a careful computation of the volume of the resting nuclei and of individual chromosomes in the early cleavage stages, and in the blastula and gastrula of Strongylocentrotus. She finds that the chromosomes of the pluteus period have only about one-fortieth the volume of those of the first spindle, but though the individual chromosomes grow smaller continually, the total nuclear volume increases at the expense of the plasma up to the late blastula stage.

1. *Nuclear growth during the cleavage of the egg of Crepidula.* The maximum, minimum and mean volumes of the nuclei at different stages of the cleavage of *Crepidula plana* are given in tables 3 to 5 and the coefficients of growth of all the nuclei are given in table 8. It remains only to summarize the facts there presented and to give the nuclear volumes and the rate of growth in certain later stages of the cleavage. This has been done in table 9, where the maximum, minimum and mean nuclear volumes of every nucleus from the 2-cell to the 32-cell stage is given, together with the coefficient of growth for each stage. Since this table starts with the 2-cell stage the coefficients of growth are different from those given in table 8, where subsequent stages are compared with the germinal vesicle or with the germ nuclei. For the purpose of determining the usual rate of growth for each cycle of cell division during the cleavage it is desirable to start with the 2-cell stage. The germinal vesicle is an extraordinarily large nucleus, and since two nuclei are present in the egg before the first cleavage the nuclear condition at this stage is unusual; on this account the rate of nuclear growth during cleavage is

TABLE 9

*Rate of nuclear growth during the cleavage of Crepidula plana*

STAGE	BLASTOMERES	MAXIMUM NUCLEAR VOLUMES	COEFFICIENT OF GROWTH	MEAN NUCLEAR VOLUMES	COEFFICIENT OF GROWTH	MINIMUM NUCLEAR VOLUMES	COEFFICIENT OF GROWTH
		<i>cubic <math>\mu</math></i>		<i>cubic <math>\mu</math></i>		<i>cubic <math>\mu</math></i>	
2 cells	AB, CD.....	14,476	1.0	6,110	1.0	381	1.0
4 cells	A, B, C, D.....	23,100	1.6	8,560	1.4	602.4	1.58
8 cells	1A-1D.....	19,396		7,068		338.4	
	1a-1d.....	5,748		3,620		338.4	
16 cells		25,144	1.73	10,638	1.74	676.8	1.77
	2A-2D.....	12,220		7,068		338.4	
	2a-2d.....	7,068		3,620		338.4	
	1a <sup>1</sup> -1d <sup>1</sup> .....	3,620		1,528		235.2	
	1a <sup>2</sup> -1d <sup>2</sup> .....	720		452		235.2	
		23,628	1.63	12,068	2.07	1147.2	3.01
24 cells	3A-3D.....	8,580		5,748		150.0	
	3a-3d.....	5,748		3,620		150.0	
	2a <sup>1</sup> -2d <sup>1</sup> .....	5,748		3,620		150.0	
	2a <sup>2</sup> -2d <sup>2</sup> .....	5,748		3,620		150.0	
	1a <sup>1</sup> -1d <sup>1</sup> .....	3,620		1,528		235.2	
	1a <sup>2</sup> -1d <sup>2</sup> .....	720		452		235.2	
		30,164	2.08	18,588	3.04	1070.4	2.80
32 cells	4A-4D.....	12,220*		7,068		171.3	
	4d.....	697		382		58.8	
	4a-4c.....	2,715		1,146		112.5	
	3a-3d.....	5,748		3,620		150.0	
	2a <sup>1</sup> -2d <sup>1</sup> .....	5,748		3,620		150.0	
	2a <sup>2</sup> -2d <sup>2</sup> .....	5,748		3,620		150.0	
	1a <sup>1</sup> -1d <sup>1</sup> .....	3,620		452		150.0	
	1a <sup>2</sup> -1d <sup>2</sup> .....	2,065		3,620		150.0	
	1a <sup>3</sup> -1d <sup>3</sup> .....	720		452		235.2	
		39,311	2.71	23,980	3.92	1327.8	3.48
Total growth in thirty divisions.....		24,835	2.715	17,870	3.92	946.8	3.48
Average growth for each division.....		827.8	1.05(=5%)	595.6	1.09(=9%)	31.5	1.08(=8%)

\* This volume is reached only at a much later stage, shortly before the closure of the blastopore (fig. 6).

During this same period from the 2-cell to the 32-cell stage the coefficient of growth of maximum nuclear surfaces is 4.28, or an average increase of about 11 per cent for each division.

best determined by comparing subsequent stages with the 2-cell stage. Furthermore the nuclear volume in the 2-cell stage is less than at any other stage, and it consequently forms a good starting point for the study of nuclear growth.

*Finally, the volume of all the nuclei in the 70-cell stage, without attempting to determine the maximum volume of each nucleus, is shown in table 10.*

At the 70-cell stage the ectomeres are already closing over the yolk on the oral hemisphere, and it may be assumed that the cleavage will show no new tendencies as to the growth of nuclear substance until the embryo as a whole begins to grow.

Whether nuclei are measured at either their maximum size, their minimum size or at a size intermediate between these two extremes, the rate of growth during cleavage is found to fall far short of a doubling or increase of 100 per cent at each division. The average nuclear growth during early cleavage is not more than 5 to 9 per cent for each division, and in the later cleavage it falls as low as 1 per cent for each division. A growth of nuclear substance at this rate scarcely deserves to be designated as 'phenomenal' or 'colossal.' On the other hand, the protoplasm which is generally supposed to remain fixed in quantity during cleavage, increases at a more rapid rate than the nuclei, from the 1-cell to the 24-cell stages, as shown in table 8. In view of the facts here presented, even though it be for only a single species, the generally accepted conclusion as to the great increase of nuclear substance during cleavage, as contrasted with the lack of growth of the protoplasm, evidently needs revision, as do also the theories which have been founded upon this supposed fact.

*2. Nuclear growth during the cleavage of the egg of Fulgur.* While my results are based largely upon the study of *Crepidula plana* they are not limited entirely to this species. The following measurements of the nuclei of *Fulgur carica* are probably not very accurate since they had to be made under a relatively low power objective (8 mm. apochromat) and since the material at my command did not permit the study of a large number of eggs, and the selection of nuclei at maximum size. Never-

TABLE 10

*Actual nuclear diameters and volumes in the 70-cell stage of Crepidula plana*

BLASTOMERE	NUCLEAR DIAMETER	TOTAL NUCLEAR VOLUME	COEFFICIENT OF GROWTH	
			Nuclear volume	Nuclear surfaces
	$\mu$	$\mu$		
2 cell stage.....	24	14,476	1	1
11 Entomeres {	4A-4D.....	16	8,579	
	4a-4c.....	10	1,571	
	E <sup>1</sup> , E <sup>2</sup> .....	7	359	
	e <sup>1</sup> , e <sup>2</sup> .....	6	226	
4 Mesomeres {	M <sup>1</sup> , M <sup>2</sup> .....	10	1,047	
	m <sup>1</sup> , m <sup>2</sup> .....	9	763	
55 Ectomeres :	<i>First quartet</i>			
	4 Apicals, 1a <sup>1-1</sup> -1d <sup>1-1</sup> .....	10	2,094	
	3 Basals, 1a <sup>1-2-1</sup> -1c <sup>1-2-1</sup> .....	9	1,145	
	1 Basal, 1d <sup>1-2</sup> .....	12	905	
	3 Middles, 1a <sup>1-2-2</sup> -1c <sup>1-2-2</sup> .....	12	2,714	
	4 Turrets, 1a <sup>2</sup> -1d <sup>2</sup> .....	7	718	
	<i>Second quartet</i>			
	3 Tip cells, 2a <sup>1-1</sup> -2c <sup>1-1</sup> .....	6	339	
	1 Tip cell, 2d <sup>1-1</sup> .....	10	524	
	4 Girdle cells, 2a <sup>1-2-1</sup> -2d <sup>1-2-1</sup> .....	9	1,527	
	4 Girdle cells, 2a <sup>1-2-2</sup> -2d <sup>1-2-2</sup> .....	10	2,094	
	4 Girdle cells, 2a <sup>2-1-1</sup> -2d <sup>2-1-1</sup> .....	9	1,527	
	4 Girdle cells, 2a <sup>2-1-2</sup> -2d <sup>2-1-2</sup> .....	9	1,527	
	4 Girdle cells, 2a <sup>2-2</sup> -2d <sup>2-2</sup> .....	5	262	
	<i>Third quartet</i>			
	4 Girdle cells, 3a <sup>1-1</sup> -3d <sup>1-1</sup> .....	10	2,094	
	4 Girdle cells, 3a <sup>1-2</sup> -3d <sup>1-2</sup> .....	9	1,527	
	4 Girdle cells, 3a <sup>2-1</sup> -3d <sup>2-1</sup> .....	6	452	
	4 Girdle cells, 3a <sup>2-2</sup> -3d <sup>2-2</sup> .....	6	452	
70 cells. Total nuclear volume.....		32,416	2.24	5.30

The total volume of these 70 nuclei is almost exactly the same as the volume of the germinal vesicle, about 50 per cent more than the volume of the germ nuclei, and 35 per cent more than the mean nuclear volume of the 32-cell stage, with which mean volume, rather than with the maximum, this actual volume of the nuclei of the 70-cell stage should be compared. In the 38 nuclear divisions leading from the 32-cell stage to the 70-cell stage the nuclear material has increased at an average rate of less than 1 per cent for each division.

TABLE 11  
*Diameters and volumes of the nuclei, 2-cell to 16-cell stages of Fulgur carica*

STAGE	BLASTOMERES	DIAMETER OF NUCLEUS	TOTAL VOLUME OF NUCLEI	COEFFICIENT OF NUCLEAR GROWTH
		$\mu$	<i>cubic <math>\mu</math></i>	
2 cells	AB, CD.....	40	67,020	1.0
4 cells	A, B, C, D.....	32	68,628	1.02
8 cells	1A-1D.....	32	68,628	1.15
	1a-1d.....	16	8,576	
			77,204	
12 cells	2A-2D.....	32	68,628	1.28
	2a-2d.....	16	8,576	
	1a-1d.....	16	8,576	
16 cells			85,780	1.40
	3A-3D.....	32	68,628	
	3a-3d.....	16	8,576	
	2a-2d.....	16	8,576	
	1a-1d.....	16	8,576	
			94,356	

theless they indicate the general rate of nuclear growth in this prosobranch.

In fourteen nuclear divisions there has been an increase in nuclear substance of 40 per cent, or an average increase for each division of 2.8 per cent. The rate of nuclear growth is practically the same in the other species of *Crepidula* as in *C. plana*; and in all prosobranchs the nuclear material increases but slightly during the cleavage period.

3. *Nuclear growth during the cleavage of other animals.* From a casual examination of the segmenting eggs of nematodes, echinoderms, amphioxus and ascidians, as well as from a study of the figures of various authors, it is evident that the nuclear growth in these forms is greater during the early cleavages than in the gastropods. In all of these forms the germinal vesicle is relatively much larger and the egg and sperm nuclei much smaller than in the gastropods, while the decrease in nuclear size in the early

cleavages is not so marked as in the gastropods, though of necessity the nuclei must grow smaller in all animals as cleavage progresses.

In the ascidian, *Styela* (*Cynthia*) *partita*, the maximum nuclear diameters and volumes in the different cell generations are shown in table 12:

TABLE 12  
*Maximum nuclear diameters and volumes in Styela (Cynthia) partita*

STAGE	AVERAGE DIAMETER NUCLEUS	TOTAL VOLUME OF NUCLEI	COEFFICIENTS OF GROWTH			
			Nuclear volume			Nuclear surfaces
	$\mu$	cubic $\mu$				
Before first ma- turation.....	54	82,448	1.0			
Before first cleavage.....	♀ 12+♂ 12	1,809	0.02	1.0		
2 cells.....	16 $\mu$	4,289	0.05	2.37	1.0	1.0
4 cells.....	14	5,748	0.06	3.17	1.34	
8 cells.....	13	9,203	0.11	5.08	2.14	
16 cells.....	11	11,173	0.13	6.17	2.60	
32 cells.....	10	16,755	0.20	9.26	3.90	
64 cells.....	8	17,152	0.20	9.48	4.00	
128 cells.....	6.5	18,406	0.22	10.17	4.29	
256 cells.....	5.25	19,395	0.23	10.72	4.52	13.75

The nuclei of different blastomeres of the same generation vary considerably in size, and I have not attempted to measure each individually, as in the case of *Crepidula*, nevertheless the measurements given represent approximately the average nuclear diameters for each generation of blastomeres. When the cells become very numerous a very slight error in the measurement makes a big difference in the results, and the total nuclear volume in the later stages may not be very accurate. Nevertheless the table does give a true idea of the order of magnitude of the nuclei in the different generations.

In comparing this table with those for *Crepidula* it will be seen at once that the germinal vesicle is relatively larger, the germ nuclei smaller and the growth of the nuclear material in the early stages greater in *Styela* than in *Crepidula*. The volume of the egg and sperm nuclei represents a loss of 98 per cent as com-

pared with that of the germinal vesicle; and even in the 256-cell stage the volume of all the nuclei is 77 per cent less than that of the germinal vesicle. Comparing the nuclear volumes of subsequent stages with that of the germ nuclei, we find that up to the 32-cell stage there is an increase of 826 per cent, or an average for the first 31 nuclear divisions of 26 per cent for each division; from the 32-cell stage to the 256-cell stage there is an increase of 146 per cent, or an average increase of 0.6 per cent for each division. Since the germinal vesicle is unusually large and the germ nuclei unusually small, a better idea of the rate of nuclear growth in the egg will be obtained by comparing the nuclear volumes of later stages with that of the two cell stage, as was done in the case of *Crepidula*. Such a comparison is given in the last column of Coefficients in table 13. From this it appears that the nuclear growth from the 2-cell stage to the 32-cell stage is 290 per cent or an average increase for each of 30 divisions of 9.6 per cent; from the 32-cell stage to the 256-cell stage the nuclear volume increases 62 per cent, or an average increase for 224 divisions of 0.27 per cent for each division.

In the cleavage of the eggs of amphioxus and of echinoderms the rate of nuclear growth is essentially similar to that of the ascidians. Here also the germinal vesicle is very large and the total volume of the nuclei at the close of cleavage is much less than the volume of the germinal vesicle, though decidedly greater than the volume of the germ nuclei at the beginning of cleavage. In all of these cases the nuclei in the early cleavages contain little chromatin and much achromatin; while they are more densely chromatic in the later stages, showing that the chromatin has increased in quantity relatively more than the achromatin. This is probably due to the fact that the chromosomes take up less cytoplasmic substance in the smaller cells than in the larger ones, the amount of achromatin in the nucleus depending in large part upon the quantity of cytoplasm in the cell.

4. *Growth of different nuclear constituents.* a. Nuclear sap. All of the substances within a nucleus do not increase at the same rate. The most abundant constituent of a fully formed nucleus is nuclear sap, and this is scarcely present at all in the earliest stages of the nuclear cycle. During each resting period the nu-

clear sap increases in amount from zero until it forms the principal bulk of the nucleus, and when mitosis comes on it passes into the cell body, and as a constituent of the nucleus sinks again to zero. The substance which forms the nuclear sap is absorbed by the nucleus from the cell body throughout the whole of the resting period, only to be thrown out into the cell body again at the end of that period. Consequently the nuclear sap is no more a nuclear constituent than a protoplasmic one, belonging to both nucleus and protoplasm.<sup>2</sup> Studies on the growth of nuclear material should therefore be confined to the growth of the chromatin, but the difficulty of measuring the amount of chromatin at different stages will be appreciated without further comment. Also the fact that so large a part of the nuclear material belongs also to the protoplasm should be taken into account in experiments dealing with the isolation of nuclei from protoplasm; evidently the only satisfactory way in which such isolation can be accomplished is by isolating chromosomes, rather than resting nuclei.

There is good reason for believing that the nuclear sap contributes to the nourishment and growth of the chromatin and linin, and that it in turn receives substances from these, so that the materials which pass into the cell body when the nuclear membrane dissolves, are not wholly the same as those which were taken up by the nucleus from the cell body. I have elsewhere ('02) called attention to the fact that the escaping nuclear sap stains more deeply than the cell protoplasm and may therefore be called 'chromatic sap.'

As to the mechanism of this intake of protoplasmic substance into the nucleus there is every visible evidence that it is of the nature of osmosis. The nucleus becomes spherical in shape unless subjected to outside pressure, or to the action of substances which cause plasmolysis. The nuclear membrane remains entire and distinct until the last phase of nuclear growth, immediately preceding mitosis, when the nucleus swells very rapidly and the nuclear membrane becomes thin and then disappears.

The measurements given in the preceding section show that the total quantity of the more fluid part of the nucleus, the nuclear

<sup>2</sup>Watake (1893) says,—"The structure known as the nucleus contains a great deal of cytoplasmic substance."



sap, does not increase in quantity during the cleavage of the egg; we have seen that the total volume of all the nuclei of *Crepidula* at the 70-cell stage is about equal to that of the germinal vesicle, while in *Styela* the volume of all the nuclei at the 256-cell stage is 77 per cent less than the volume of the germinal vesicle. The conclusion is justified, therefore, that the more fluid constituent of the nucleus decreases greatly in volume during the early cleavage stages, and that the nuclei therefore become denser during this period.

b. Linin. Just as the nuclear sap is proportional in volume to the volume of the nucleus as a whole, so also it is evident that the linin is more abundant in large nuclei than in small ones. Evidently it is not possible to determine the volume of linin in a resting nucleus, but since the spindle fibers are composed largely of linin it is possible by measuring the size of spindles to determine, at least in a general way, the relative quantities of linin in different nuclei. In the following table the length of the spindle from

TABLE 13  
*Length of spindle in the maturation and cleavage of Crepidula plana*

STAGE	LENGTH OF SPINDLE	DIAMETER OF PRECEDING NUCLEUS
	$\mu$	$\mu$
First maturation.....	42	42
Second maturation.....	18	—
First cleavage.....	30	34.5
Second cleavage, AB, CD.....	30	24
Third cleavage, A, B, C, D.....	27	22
Fourth cleavage, 1A-1D.....	25	21
Fourth cleavage 1a-1d.....	21	12
Fifth cleavage 2A-2D.....	24	18
Fifth cleavage 2a-2d.....	21	15

centrosome to centrosome is given for successive cleavages of *C. plana*, the measurements being made in each case in the stage of the metaphase. The diameter of the nucleus is also given for comparison with the spindle length (table 13).

In general the diameter of the spindle at its equator is, in the prophase and metaphase, about the same as the diameter of the nucleus from which it came. Spindles in the protoplasmic ectomeres are relatively larger than the size of the nucleus would lead

one to expect and this probably is due to the fact, which I ('05) have established in the ascidians, that the polar parts of the spindle are not derived from the nucleus but from the protoplasm. With this proviso, it is true that, within the same species, large nuclei give rise to larger spindles than do small ones and this may be held to indicate that the linin is more abundant in the former than in the latter.

The fact that the spindle fibers of ascidians are composed of equatorial and polar parts, the former derived from the nucleus and the latter from the protoplasm, and the fact that these two portions of the spindle, and also the polar fibers, are fundamentally alike, indicates that the linin, like the nuclear sap, is a constituent which belongs both to the nucleus and to the protoplasm.

c. Chromatin. The amount of chromatin undoubtedly increases during the cleavage; the resting nuclei in the later stages being more densely chromatic than those of the earlier stages. In each cell the chromatin is smallest in quantity when the daughter chromosomes are first separated, and it grows in quantity during the resting period. Not all of the chromatin of the resting stage goes into the formation of the chromosomes of the next mitosis, but some of it in the form of granules (oxychromatin) or chromatic sap escapes into the cell body on the dissolution of the nuclear membrane. The larger the nucleus is and the longer the resting period through which it has come, the greater the quantity of chromatin which thus escapes at mitosis. Gardiner ('98) estimated that the amount of chromatin which thus escaped into the cell body at the first maturation division of *Polychaerus* was five hundred times as great as that which went to form chromosomes, and conditions are similar in *Styela*, *Crepidula*, and many other forms. Consequently the volume of the chromosomes in successive stages cannot be used as a measure of the growth of the chromatin. Nevertheless the growth of the chromosomal mass, as well as the growth of the entire nuclear volume, will give some idea as to the growth of the chromatin during cleavage. Table 9, giving as it does the volumes of the nuclei and chromosomal plates at various stages, furnishes data upon which an opinion as to the growth of the chromatin of the resting stages

may be based. From the 2-cell to the 32-cell stages the growth in volume of the resting nuclei lies between 171 per cent for maximum nuclear size, and 292 per cent for mean nuclear size while the growth of the chromosomal plates is 248 per cent. It seems very probable therefore that the growth of the chromatin during these stages lies somewhere between 171 per cent and 292 per cent, or an average increase for each of the 30 divisions represented of from 5.7 per cent to 9.7 per cent. In all cases the growth of the chromatin falls far short of 100 per cent, or a doubling, in each division cycle. In *Strongylocentrotus*, Erdmann ('08) finds that the ratio of chromatin to plasma is seven times greater in the pluteus than at the beginning of development, and she points out that this means that plasma contributes to the growth of the chromatin.

While the chromatin as such is peculiar to the nucleus, there can be no doubt that large quantities of chromatin escape into the protoplasm. Such chromatin usually loses its distinctive staining reaction and presumably suffers chemical change. On the other hand we know that chromatin grows at the expense of substances received from the protoplasm. The work of Masing ('10) on the nucleic acid content of the egg indicates that this important constituent of chromatin is about as abundant in early stages as in later ones; he supposes that it exists in the protoplasm.

d. Chromosomes. What is true of the quantitative relations of the chromatin as a whole is true also of the individual chromosomes; those formed from large nuclei are larger than those from small ones; the chromosomes do not double in volume in each successive cleavage, but they become individually smaller as cleavage progresses. These facts are not difficult to demonstrate, but they are difficult to express in any numerical proportion, owing to the irregular shape and small size of the chromosomes, which make it very difficult to determine their volume.

In *Crepidula* the chromosomes are very small and numerous, the full number being probably 60, and they are usually crowded together so that it is difficult to photograph them, or even to draw their outlines accurately, and since they are so small it is

not practicable to measure them directly with the 1/1 micrometer eyepiece. Nevertheless by selecting sections in which only a part of the chromosomes are shown I have been able to sketch the outlines of many of them with what I believe to be substantial accuracy. For the purpose of comparing the sizes of chromosomes from different cleavages I have chosen two generations of blastomeres in which the difference in the size of the nuclei is at a maximum, the nucleus in one cell being about twice the diameter of that in the other; these blastomeres are the macromeres *AB* and *CD*, and the micromeres *1a-1d* (figs. 7 and 8). In the former the diameter of the nucleus just before division is about  $24\mu$ , in the latter about  $14\mu$ . When the nuclei of the cells in question had begun to divide and the mitotic figures were in the equatorial plate stage, the chromosomes from a number of these spindles were drawn as accurately as possible with a camera lucida. In order to be certain that the stage of division was the same in each case only longitudinal sections through the spindle were chosen; and in order to avoid as far as possible individual differences in the sizes of chromosomes, only the largest and most isolated chromosomes were drawn. Fig. 9 shows chromosomes from four different spindles of the second cleavage; fig 10 shows chromosomes from the first division of the first quartet cells (*1a-1d*), also from four different spindles. In all cases the chromosomes are magnified 2000 diameters.

It is plain from these figures that the chromosomes from the larger nuclei are larger than those from the smaller ones, though the difference in the diameters and volumes of the chromosomes are not as great as the difference in the volumes of the nuclei from which they came. The average volume of the chromosomes from the large nuclei is about 5.2 cubic  $\mu$  and of those from the small nuclei about 2.6 cubic  $\mu$ . While the volumes of the nuclei as a whole are to each other about as 5 : 1, the volumes of their individual chromosomes are to each other as 2 : 1. In the case of nuclei which differ but slightly in volume it is not possible to be certain that the chromosomes differ in size, but in all cases in which the differences in the size of nuclei is considerable it can

be seen that the larger nuclei give rise to larger chromosomes than do the smaller ones.

Since the probable error is much greater in the measurement of individual chromosomes than of whole chromosomal plates, I have not attempted to measure individual chromosomes in each stage of the cleavage; on the other hand the dimensions of the chromosomal plates are given in table 4 for each cell up to the 32-cell stage. These measurements show that from the 2-cell to the 32-cell stage the chromosomal mass increases in volume 248 per cent or an average of 8 per cent for each of 30 divisions. The chromosomal plates, and consequently the individual chromosomes, grow smaller as cleavage advances, but in the same generation of cells small nuclei have smaller chromosomes than large ones. In short, the size of the chromosome is dependent upon the size of the nucleus from which it comes, rather than upon the cell generation to which it belongs.

In the main these observations are in harmony with those of Erdmann, and Baltzer, to which reference has already been made. In *Crepidula*, as in the echinids studied by the authors named, the individual chromosomes grow smaller as the cleavage advances, but this is causally related to the decrease in the size of the nuclei and of the cells, and where, in later cleavage stages, the nuclei and cells remain large, there the chromosomes also are larger than in smaller sister cells. Just as the size of the nucleus is connected with the volume of the cytoplasm in which it lies, so the size of the chromosomes is connected with the volume of the nucleus from which they come.

Montgomery ('10) has found that the sperm cells of *Euschistus* are of two sizes and he concludes (p. 127), that "it is probable that the large sperm possess no more chromatin than the small, though the heads in the former are much larger. The dimегaly expresses itself accordingly in differences of amount of karyolymph and of the substance (linin) that composes the mantle fibers, but much more markedly in the amount of cytoplasm." He finds also that the mitochondria (idiozome) increase directly with the amount of cytoplasm. According to my observations chromosomes from large nuclei are larger than those from small ones of

the same generation, though naturally it is more difficult to detect size differences in objects as small as chromosomes than in entire nuclei. Where the differences in nuclear volumes are great one can always detect corresponding differences in chromosome volumes.

The chromosomes of the spermatid are usually smaller than those of the oötid, but when the chromosomes of the first cleavage spindle appear, those from the sperm nucleus are usually as large as those from the egg. The reason for this is to be found in the fact that both grow, after fertilization, in the same medium, the egg plasma, and for approximately the same length of time.

e. Plasmasomes. The conclusion that large nuclei have large chromosomes, and *vice versa*, also applies to the sizes of nucleoli (plasmasomes); they are larger in large nuclei than in small ones. However in this case another factor is involved for the size of nucleoli is not only dependent upon the size of the nucleus, but also upon the length of the resting period; indeed the latter seems to be the more important factor of the two. The largest of all nucleoli is the one found in the germinal vesicle, at the close of the longest resting period in the entire life cycle. In these gasteropod eggs the next largest nucleoli are found in the cells 4A-4D and 4a-4c (fig. 6) in which the resting stage is particularly long. The nuclei of the cells 4A-4D are of the same size as those of 2A-2D viz.  $18\mu$  in diameter, but the nucleoli of the former have about three times the diameter of those of the latter.

In earlier stages of cleavage where the blastomeres are dividing rapidly it is difficult to compare the sizes of nucleoli, not only because their number varies considerably, but also because each plasmasome is usually surrounded by a layer of chromatin granules which renders exact measurements difficult. The number of plasmasomes appears to depend to a large extent upon the degree of fusion of an originally large number of separate plasmasomes. When chromosomes are isolated so that each gives rise to a distinct vesicle, each may contain a minute plasmasome, and there may be as many of these as there are chromosomal vesicles. In *Crepidula* the number is always greatest during the earlier stages of the resting period; during the later stages they appear to fuse

together becoming fewer and larger as the individual chromosomal vesicles fuse. For a considerable period two nucleoli are commonly found in each nucleus, one in each gonomere, or nuclear half. However, when the resting stage is long, these two fuse into a single large plasmasome.

While the nucleus continues to grow in size up to the time of the dissolution of the nuclear membrane, the plasmasome usually disappears before the formation of the spireme. In comparing the relative sizes of nucleoli it is important to compare corresponding stages; accordingly in my measurements they were measured when they had reached approximately their maximum size, and before the nucleus had reached its maximum. Nucleoli differ more or less in size even in different cells of the same generation, owing perhaps to the more or less complete fusion of the many original nucleoli; it is significant in this connection that after a long resting period they are much more uniform in size and constant in number than when the resting period is short. The following table gives the diameters of nuclei and nucleoli (plasmasomes) in various blastomeres of *Crepidula*:

TABLE 14  
*Maximum nucleolar size and nuclear size in the blastomeres of Crepidula plana*

STAGE	BLASTOMERES	DIAMETER OF NUCLEUS	VOLUME OF NUCLEUS	NUMBER OF NUCLEOLI	DIAMETER OF NUCLEOLI	VOLUME OF NUCLEOLI	NUCLEAR- NUCLEOLAR RATIO
		$\mu$	cubic $\mu$		$\mu$	cubic $\mu$	
1 cell, before maturation		42	32,409	1	12	905.0	35 : 1
2 cells, AB, CD		20	4,189	2	3	28.0	149 : 1
4 cells, A, B, C, D		15	1,767	2	2½, 1½	7.7	220 : 1
8 cells	{ 1A-1D	19	3,591	2	3	28.0	128 : 1
	{ 1a-1d	13	1,150	2	2, 1½	7.0	164 : 1
12 cells	{ 2A-2D	14	1,437	2	2	8.3	180 : 1
	{ 2a-2d	15	1,767	2	2	8.3	220 : 1
16 cells	{ 1a¹-1d¹	12	905	2	3, 2	18.3	50 : 1
	{ 1a²-1d²	7	180	2	1	1.0	180 : 1
20 cells	{ 3A-3D	15	1,767	1	7½	221.0	8 : 1
	{ 3a-3d	15	1,767	2	3	28.0	63 : 1
25 cells	4d	9	382	1	3	14.0	27 : 1
32 cells, 4a-4c		13	905	1	6	113.0	8 : 1
Ca. 100 cells, 4A-4D		15	1,767	1	9	382.0	4.6 : 1
Fulgur carica:							
Ca. 1000 cells, 4A-4D		96	462,192	1	27	10306.0	44 : 1

In eggs in which the nuclear division has been greatly delayed, if not entirely stopped, by the use of hypertonic salt solutions the nucleoli become much larger than in normal eggs. Thus in the eggs of *Crepidula plana* treated with 4 per cent NaCl solution for two hours, and then put into normal sea water for six hours, the sizes of nuclei and nucleoli are as follows:

TABLE 15

*Nucleolar size and nuclear size in eggs of Crepidula plana in hypertonic sea water*

STAGE	BLASTOMERES	DIAMETER OF NUCLEUS	VOLUME OF NUCLEUS	NUMBER OF NUCLEOLI	DIAMETER OF NUCLEOLI	VOLUME OF NUCLEOLI	NUCLEAR- NUCLEOLAR RATIO
		$\mu$	cubic $\mu$		$\mu$	cubic $\mu$	
1 cell, pronuclei.....	♀	24	7,238	1	12	905	8 : 1
	♂	21	4,849	1	10	524	9 : 1
2 cells, AB, CD.....		24	7,238	1	9	382	19 : 1
4 cells, A, B, C, D.....		15	1,767	1	9	382	4.6 : 1
8 cells, 1A-1D.....		18	3,055	1	9	382	8 : 1

The great size of the single nucleolus in each of these nuclei is probably due to the fact that division has been delayed and the resting period prolonged.

f. Centrosomes and spheres. Finally we may consider in this connection the sizes of centrosomes, and spheres though they are not parts of the nucleus. In general in *Crepidula*, large cells contain large centrosomes and spheres, while small cells contain small ones. The maximum diameters of centrosomes in the cleavage of *C. plana*, vary from  $2\mu$  to  $7\mu$ , the measurements being made during the telophase of division. The maximum diameters of the sharply defined spheres, during the resting stages, vary from  $5\mu$  to  $12\mu$ ; and in all cases, so far as I have observed, the largest centrosomes and spheres occur in the cells which have the largest amount of protoplasm, while the smallest occur in the cells with the least amount of protoplasm.

The centrosomes and spheres are the cell constituents which first become unequal in an unequal cell division. As soon as the spindle becomes eccentric, the centrosome and sphere which lies farthest from the center of the cell becomes smaller than the one



at the opposite pole. Only after the division wall forms do the daughter nuclei become unequal.

5. *Conclusions as to nuclear growth during cleavage.* The rate and amount of nuclear growth during cleavage is much less than is generally believed. Whether the nuclear volume is taken when the nuclei are at their maximum, mean, or minimum size, the nuclear growth is far from 100 per cent, or a doubling, in each division. In *Crepidula* the nuclear growth is not more than 5 per cent to 9 per cent for each division from the 2-cell to the 32-cell stage, and less than 1 per cent for each division after the 32-cell stage. At the 2-cell stage the nuclear volume is least and up to the 32-cell stage the chromatin increases at an average rate of about 8 per cent for each division. The stage when the volume of protoplasm is least, after the egg has reached its full size, is just before the first maturation division; between the first maturation and the 24-cell stage the protoplasm increases at an average rate of nearly 6 per cent for each division. At the end of cleavage the ratio of nuclear material to protoplasmic differs but little from the ratio at the beginning. In *Fulgur* the nuclear growth from the 2-cell stage to the 16-cell stage averages only 2.8 per cent for each division, and the general Kernplasma-Relation remains unchanged. In *Styela* the nuclear growth from the 2-cell to the 32-cell stage averages 9.6 per cent for each division; from the 32-cell stage to the 256-cell stage it averages only 0.27 per cent for each division. Such a rate of growth is not significant and indicates that the meaning of cleavage is to be found in something other than the increase of nuclear material as compared with the plasma.

In general the growth of each of the different nuclear constituents parallels the growth of the nuclear material as a whole, though this is not true of the nuclear sap, which belongs to both cytoplasm and nucleus. During cleavage the fluid content of the egg as a whole decreases, the oöplasm becoming more consistent in later stages than in earlier ones. The total fluid content of the nuclei in the early cleavage stages is much less than that of the germinal vesicle; even in the later cleavages the nuclear sap is not so abundant, in some animals, as in the germinal vesicle. In *Crepidula* the volume of all the nuclei at the 70-cell stage is

only equal to that of the germinal vesicle, though the volume of the chromosomal plates has increased 250 per cent; in *Styela* the volume of all the nuclei of the 256-cell stage is 77 per cent less than that of the germinal vesicle, though the total chromosomal volume has increased many fold during this period.

Linin is a nuclear constituent which is found also in the protoplasm, and during cleavage it grows in quantity at about the same rate as the nuclear and protoplasmic materials as a whole. The polar parts of the spindle and the astral rays arise in the protoplasm outside the nucleus, while the equatorial portion of the spindle comes from the nucleus, as is shown with great clearness in the cleavage mitoses of ascidians. Correspondingly the size of the spindle is a resultant of the volume of the nucleus and of the protoplasm.

Chromatin is more distinctively a nuclear substance than the nuclear sap or linin, though it undoubtedly grows at the expense of substance received from the protoplasm and in turn contributes material to the protoplasm. From the 2-cell to the 32-cell stage in *Crepidula* the growth, of the chromatin amounts to between 6 per cent and 10 per cent for each division, and as the fluid contents of the nuclei do not increase during cleavage the nuclei become more chromatic in later stages than in earlier ones.

Chromosomal material, as represented in the condensed chromosomal plates of the anaphase, increases in volume 248 per cent from the 2-cell to the 32-cell stages of *Crepidula*, or an average growth of about 8 per cent for each division. Individual chromosomes grow smaller as cleavage advances, but this is due to the smaller size of the nuclei from which they come rather than to the cell generation to which they belong; nuclei of the same generation which differ greatly in size produce chromosomes which differ in size, the larger nucleus producing larger chromosomes than the smaller one.

In the blastomeres of *Crepidula* the size and number of nucleoli (plasmosomes) are influenced by the size of the nucleus and the length of the resting period. In most of the nuclei there are two nucleoli, but when the resting period is long, these fuse into a single one. In experiments, anything which prolongs the resting

period leads to an increase in the size of the nucleoli. During the normal cleavage of *Crepidula* the ratio of the nuclear volume to the nucleolar volume varies from 220 : 1 to 4.6 : 1.

Centrosomes and spheres are proportional in size to the volume of the protoplasm in which they lie; they are always larger in large cells than in small ones and hence they grow progressively smaller as cleavage advances.

In general the volume of each of the nuclear constituents named is influenced by the volume of protoplasm of the cell, and by the length of the resting period. The protoplasm contributes substances to the growth of each of these constituents, and the more abundant it is the larger they grow, provided the period of growth is the same in all cases. Where the growth period (interkinesis) is very long the nuclei becomes unusually large and may ultimately absorb the greater part of the protoplasm.

6. *Comparison of growth of chromatin with increase of chemical substances and processes during cleavage.* Loeb in several important papers has shown that the nucleus is the oxidizing center of the cell, and that the chromatin is chiefly concerned in bringing about oxidations. Warburg ('08) found the oxidative power of the egg to increase at a relatively slow rate during cleavage. More recently, in view of the oft-repeated assertion that the chromatin doubles at each division, Loeb ('09) concluded that the supposed growth of chromatin in geometric ratio indicates that nuclear synthesis is of the nature of an autokatalytic reaction. Masing ('10) has shown that in the eggs of *Arbacea pustulosa* the nucleic acid in the fertilized but unsegmented egg is as great as in the 'morula' with 500 to 1000 cells. He concludes that, "the colossal increase of nuclear mass in the cleavage leads to no perceptible increase of nucleic acid in the germ. A corollary of this must be that the total quantity of nucleic acid necessary to build up the nuclear apparatus of the germ must be preformed in the protoplasm" (quoted from Godlewski, '11). Shackell ('11) has reached a similar conclusion with regard to the nucleic content of the egg and blastula of *Arbacea punctulata*.

The results of my observations as to the rate of the growth of chromatin is especially significant when compared with the work

of Warburg. I find that the chromosomal mass grows at the rate of 8 per cent for each division up to the 32-cell stage. It is difficult to connect this rate of growth of the chromosomes with the lack of growth in the nucleic acid content as shown by Masing, or with the lack of growth of the nuclein content as shown by Shackell, and it seems necessary to assume as both of these investigators have done, that these substances are already pre-formed in the protoplasm. If this be true, I venture the suggestion that the large amount of chromatin (oxychromatin) which escapes into the cell body when the germinal vesicle dissolves may constitute the nuclein and nucleic acid which is distributed through the cell body.

*VII. Senescence, rejuvenescence, and the ratio of nucleus to plasma.*

It is well known that Minot ('90, '95, '08) maintains that the cause of senescence is the increase of plasma and its products at a rate greater than that of the nucleus. According to his view the egg at the beginning of development is in a senile condition, "in which there is an excessive amount of protoplasm in proportion to the nucleus, and in order to get anything which is young a process of rejuvenation is necessary . . . . During the segmentation of the ovum the condition of things has been reversed so far as the proportions of nucleus and protoplasm are concerned. We have nucleus produced, so to speak, to excess. The nuclear substance is increased during the first phase of development. Hence our conclusion:—Rejuvenation is accomplished chiefly by the segmentation of the ovum." He sums up his views on this subject in his four laws of age ('08, p. 250), the first two of which are: 1. "Rejuvenation depends on the increase of the nuclei. 2. Senescence depends on the increase of the protoplasm, and on the differentiation of the cells."

Richard Hertwig's views ('89, '03, '08) are apparently diametrically opposed to those of Minot, though I do not find them so definitely expressed. He finds that senescence, or rather 'depression' and 'physiological degeneration,' are accompanied by an enormous growth of the nucleus. As a result of his work on

Actinosphaerium and Infusoria, which had been overfed for a long time, he found that there was an enormous growth of the nucleus followed by physiological degeneration. The animals which saved themselves from this condition did it by the reduction of their nuclei, either by eliminating nuclear substance directly, or by the loss of the greater part of the nuclear material during conjugation, after which normal nuclear conditions were restored. He regards the immature egg cell, with its great nucleus, as in a condition of depression similar to that found in the protozoa named. By the processes of maturation and fertilization this nuclear material is greatly reduced: "Beim Beginn der Furchung und auch später ein enormes Missverhältniss von Kern und Protoplasma vorhanden ist, und dieses Missverhältniss allmählich eine Ausgleich erfährt, indem Zellsubstanz in Kernsubstanz umgewandelt wird," ('03, p. 116). Apparently then, in Hertwig's view, senescence or depression, is accompanied by too great an amount of nuclear material, which is then reduced, by maturation in the case of the egg cell, to such an extent that this enormous disproportion of nucleus to protoplasm appears; later, by means of the process of cleavage, during which the nuclear material grows at the expense of the protoplasm, the normal relations of nucleus to protoplasm are restored.

Popoff ('08) accepts Hertwig's view in all essential respects. He adds the interesting suggestion that in their period of depression preceding maturation the sex cells are so weakened that they are unable to assimilate nutriment, and they consequently store up food as yolk. The formation of yolk, glycogen and fat are, according to this author, not indications of increased activity of cells, but of incapacity to carry the organic synthesis to its end, viz., the formation of plasma.

While Minot's hypothesis differs fundamentally from Hertwig's as to the cause of senescence, the former holding that it depends upon the increase of protoplasm over nucleus, the latter that it is accompanied by an increase of nucleus over protoplasm, both agree that in the segmentation of the egg there is an enormous growth of the nuclear material as compared with the protoplasm.

Neither Minot nor Hertwig took account of the fact that a large part of the nuclear contents belongs to both nucleus and protoplasm. The 'Kernplasma-Relation' depends very largely upon the quantity of protoplasmic material temporarily in the nucleus; in the 4-cell stage of *Crepidula* the ratio of nuclear volume to protoplasmic volume is 1 : 6.6 when the nuclei are measured at their maximum size, but 1 : 203.8 when they are measured at their minimum size. Neither of the authors named, in describing the enormous growth of the nuclear material during cleavage, took account of the growth of the protoplasm during cleavage at the expense of the yolk.

My observations on *Crepidula* have yielded the following results, which bear upon the hypothesis under discussion: (1) While the germinal vesicle is absolutely the largest nucleus in the early stages of development, it is not so large with reference to the protoplasm, and hence according to Hertwig, not in so deep a depression, as the nuclei of certain blastomeres, which *ex hypothesi* should be undergoing restoration to normal conditions. (2) The growth of nuclear material during cleavage is not nearly so great as has been assumed, averaging not more than 10 per cent for each division up to the 32-cell stage, and not more than 1 per cent for each division after that stage. (3) The growth of protoplasm at the expense of yolk during maturation and early cleavage is considerable, averaging about 6 per cent for each division up to the 24-cell stage. (4) The 'Kernplasma Relation,' while constant for specific blastomeres, is by no means uniform for all the blastomeres of a given stage, but may vary from 1 : 1 to 1 : 14 in different blastomeres of the same generation. (5) The 'Kernplasma-Relation' in adult epithelial cells of all three germ layers is about the same as in the majority of the blastomeres. (6) The absolute size of the nucleus depends upon the quantity of protoplasm in the cell and the length of the resting period (interkinesis). (7) The greater part of the nuclear volume consists of material which belongs to the protoplasm as much as to the nucleus; during the resting period this is taken in osmotically through the nuclear membrane, and is given out again at mitosis by the dissolution of that membrane. (8) The immature egg cell, which

according to Popoff is so weakened that it is unable to assimilate nutriment, and consequently can only store up food instead of making protoplasm, does as a matter of fact form protoplasm throughout the whole of the growth period.

So far as they go, therefore, these results do not support the view that senescence is due to either an increase or to a decrease of nuclear volume as compared with that of the protoplasm. But I think that this conflict between my results and those of Minot and Hertwig is, after all, confined to details, and that in the fundamental conception of the causes of senescence and rejuvenescence they may be brought into harmony. With the general thesis that senescence is associated with the accumulation in the cell of the products of metabolism and differentiation, and that rejuvenation consists in a return to a condition in which these products are largely eliminated, as Minot and Hertwig have urged, I am in hearty agreement; their assumption that changes in the nucleus-plasma ratio are the causes of these phenomena seems to me to be merely an error of detail.

In a very suggestive paper, Child ('11) has recently maintained that senescence and rejuvenescence are caused by a decrease or an increase in the fundamental metabolic reactions. Anything which decreases the rate of metabolism, such as "decrease in permeability, increase in density, accumulation of relatively inactive substances, etc.," will lead to senescence. "Rejuvenescence consists physiologically in an increase in the rate of metabolism and is brought about in nature by the removal in one way or another of the structural obstacles to metabolism" (p. 609).

This hypothesis finds much support in the phenomena connected with the early development of the egg. It is well known that constructive metabolism takes place only in the presence of nuclear material, and it has long been known that the nuclei of various kinds of gland cells give off substances which play an important part in the metabolism of the cell. Loeb ('99) has shown that the nucleus is the oxidative center of the cell; Mathews identifies oxidase with chromatin; R. Lillie ('02) finds that oxidation takes place most rapidly in the immediate vicinity of the nucleus. If the rate of metabolism is associated with sen-

escence or rejuvenescence, as Child maintains, anything which facilitates the interchange between nucleus and protoplasm should lead to rejuvenescence, anything which decreases it should lead to senescence.

During cleavage the increase in nuclear surfaces is much greater than the increase in nuclear volumes. While the increase in maximum nuclear volumes up to the 32-cell stage of *Crepidula* is about 5 per cent for each division, the growth in the maximum nuclear surfaces during this period is about 11 per cent for each division. From the 2-cell to the 70-cell stage the nuclear volume increases only 2.24 times, while the nuclear surfaces increase 5.30 times. In *Styela* the nuclear volume increases from the 2-cell stage to the 256-cell stage only 4.52 times, the nuclear surfaces increase 13.75 times. Unquestionably this greater growth of nuclear surfaces as compared with nuclear volumes, facilitates the interchange between nucleus and protoplasm. There is also a considerable increase of cell membranes during cleavage, but most of this increase is confined to surfaces of contact between cells, and free surfaces show but little growth. My observations teach that there is little, if any, interchange of materials through partition walls separating cells.

Another and much more efficient means of facilitating the interchange between nucleus and protoplasm is found in the mitotic division of the nucleus. During the cycle from one division to the next the nucleus absorbs materials from the cell body, only to throw back into the cell body these and other materials when the nuclear membrane dissolves in mitosis. The chromatin is thus brought into the most intimate relations with the protoplasm. There is thus a sort of "diastole and systole of the nucleus" (Conklin, '02), by which the interchange between nucleus and protoplasm is greatly hastened. Indeed in the paper just referred to I suggested that this function of mitosis may be quite as important as the division and separation of the chromosomes, which is usually supposed to be the one function of mitosis.

The hypothesis that the more rapid interchange between nucleus and protoplasm is associated with increased metabolism is supported by some very significant physiological work on the



maturation, fertilization and cleavage of the egg. Loeb first showed that the immature egg, with germinal vesicle intact, is metabolically inactive; it absorbs but little oxygen and gives off little carbon dioxide. On the other hand when the membrane of the germinal vesicle dissolves, metabolic activity increases, and unless the egg is started in the process of development, by fertilization or other means, it soon dies. Lyon ('04) found that during the cleavage of the sea urchin egg the evolution of carbon dioxide is more rapid during the periods of division than during those of rest.<sup>3</sup> Warburg ('08) found that the fertilized sea-urchin egg uses six to seven times as much oxygen as the unfertilized egg. It is well known that the condensed chromatin of the chromosomes is brought into intimate relation with the protoplasm during mitosis, and of course the same is true of the condensed chromatin of the sperm head following fertilization. We may conclude, I think, that mitosis increases metabolism by facilitating the interchange between nucleus and protoplasm, and particularly by setting free chromatin in the protoplasm, either by the dissolution of the nuclear membrane, or by the introduction of the sperm head in fertilization.

Rapid and intimate interchange between the chromatin and the protoplasm is the condition of rapid metabolism, and *ex hypothesi* of rejuvenescence; slow interchange is the condition of slow metabolism, and of senescence. Such a view has many points in common with the hypotheses of Minot and Hertwig, while it avoids many of the serious difficulties which those hypotheses encounter. It is thus evident that one may hold, with Minot and Hertwig, that the germ cells before maturation are senescent, and that maturation, fertilization and cleavage represent a rejuvenescence, without necessarily connecting these processes with the nucleus-plasma ratio.

<sup>3</sup>R. Lillie (1910) holds that this is due to increased permeability of the plasma membrane during division.

## PART II

EXPERIMENTAL STUDY OF CELL SIZE AND NUCLEAR SIZE IN  
THE EGGS OF CREPIDULA PLANA*I. Nuclear size and chromosome number*

In *Crepidula* the relation of nuclear size to chromosome number is the same as in the Echinid larvae studied by Boveri ('05). By the use of various hypertonic salt solutions abnormal mitoses may be produced in *Crepidula* eggs; one of the most common of these abnormalities consists in the scattering of the chromosomes, so that they do not fuse together to form two daughter nuclei, one in each cell, but many small nuclei. Indeed there may be almost as many small nuclei as there are chromosomes, every isolated chromosome being capable of producing a small nuclear vesicle. In all such cases the nuclear vesicles formed from a small number of chromosomes always remain smaller than those formed from a larger number. In any given species the size of the nucleus is proportional to the number of chromosomes which go into its formation, providing the other factors which control nuclear size, viz., quantity of cytoplasm and length of resting period, are the same. On the other hand the size of the cell body is not dependent upon the size of the nucleus in the early cleavages of *Crepidula*, as Gerassimoff ('02) found to be the case in *Spirogyra* and as Boveri determined in the case of Echinid larvae, but the reverse is true.

In the eggs of *Crepidula* which have been treated with salt solutions the cell body frequently does not divide at all and many nuclei may be left in a single cell; where the cell itself divides there is a tendency for the blastomeres to divide in normal fashion, giving rise to macromeres or micromeres as in the normal egg, even though polyasters and abnormal mitoses are present. Consequently these eggs afford no evidence that the size of the nucleus has an influence on the size of the cell body.

*II. Nuclear size and cell size in centrifuged eggs of Crepidula*

While the size relations of cells and of their various constituents may be readily observed in normal eggs, it is especially in eggs which have been centrifuged at various stages of development that the factors which determine these various size relations can be most satisfactorily studied. The various constituents of a cell may be moved by centrifugal force to one pole or another, according to their specific weights, and the axis of centrifuging. In this way the yolk, the cytoplasm, the nuclei and the centrosomes, may be caused to take very abnormal positions in the cell. Even the mitotic figure may be moved out of its ordinary position in the earliest stages of its formation, but after it has reached the metaphase it can be moved only with great difficulty; from this stage on it is anchored, probably to the cell membrane by the astral radiations, while the other constituents of the cell are free to move under the influence of centrifugal pressure. In this way it happens that the cytoplasm may be centrifuged away from the spindle and the latter left in a dense mass of yolk; or the normal relations of cytoplasm and yolk to the poles of the spindle may be completely changed; or the normal size relations of the daughter cells may be quite reversed. As illustrating these changed relations, due to centrifuging, a few eggs are shown in figs. 11-37, selected from a great number which are similar to these.

These eggs were centrifuged on a centrifugal machine run by water pressure, at the rate of 2000 revolutions per minute; the radius of rotation was 6 cm., consequently the centrifugal pressure was nearly 270 times that of gravity. Eggs were centrifuged at this rate for varying lengths of time, after which they were removed from the machine and either fixed at once, or left for a longer or shorter time in sea water before fixation. All eggs were fixed in Kleinenberg picro-sulphuric mixture, were preserved in 70 per cent alcohol only long enough to wash out the fixing fluid, and were then stained in my modification of Delafield's haematoxylin and mounted entire in balsam, in the manner described in previous papers (Conklin, '02 *et seq.*)

In fig. 11 an egg is shown which was centrifuged for ten minutes after the formation of the first polar body and before the formation of the second, the axis of centrifuging being such that the lighter protoplasm was thrown to the vegetative pole and the heavier yolk to the animal pole, thus reversing the normal positions of these substances. After centrifuging, the egg was left in sea water for three hours before being fixed. The first polar body, which has partially divided, lies at the animal pole; the second maturation spindle has been greatly elongated and its axis has been turned somewhat, its lower pole having been moved to the right in the figure. The egg has begun to constrict opposite the equator of the spindle, thus leading to the formation of a giant second polar body. The nucleus of this second polar body consists only of a compact mass of chromosomes surrounded by yolk; the sphere connecting these chromosomes with the egg membrane is much elongated. The egg nucleus and sphere at the lower pole of the spindle are in contact with the field of cytoplasm and are much larger than those at the upper pole. The sperm nucleus and sphere, lying in the cytoplasmic field, are much the largest in the egg. In normal condition these relations are reversed, the sperm nucleus lying in the yolk, while the egg nucleus is in the cytoplasmic field; and in such cases the egg nucleus and sphere are larger than those of the sperm; however as the sperm nucleus approaches the egg nucleus and thus moves up into the cytoplasm it continually grows larger until, at the time the two meet, the sperm nucleus is almost as large as the egg nucleus. The fact that the normal size relations of these two nuclei may be reversed by reversing the positions of the cytoplasm and yolk, furnishes conclusive evidence of the fact that the relative sizes of the egg and sperm nuclei and asters are dependent upon the quantity of cytoplasm in which they lie.

Furthermore fig. 11 shows that the spindle itself is a structure composed of fibers more firm than the surrounding substance, and is not merely an arrangement of the granules, which happen to be present in a field of force, into lines, like iron filings in a magnetic field. The spindle remains fixed in position when all surrounding substances change position, and the spindle fibers,

though much elongated preserve their usual appearance. In this regard my work confirms the conclusions of Morgan ('10) as to the nature of the spindle in *Cerebratulus*, and is at variance with the work of Lillie ('09) on *Chaetopterus*.

In fig. 12 an egg is shown which was centrifuged for fifteen minutes during the first cleavage and was then left for three hours in sea water. The axis of centrifuging is indicated here, as elsewhere, by the lighter vacuolated substance at one pole and the heavier yolk at the opposite pole; this axis is also marked by an arrow, the head of the arrow marking the distal pole during centrifuging, the tail of the arrow the central pole. In figs. 12 to 15 the first cleavage plane does not pass through the animal pole, which is marked by the polar bodies, but is displaced to one side, and the cleavage is not meridional, as in normal eggs; furthermore the cleavage is not equal, quantitatively and qualitatively, as in normal eggs, but is markedly unequal, most of the cytoplasm having gone into the smaller one of the two daughter cells, while the larger one contains little cytoplasm and much yolk. This is evidently due to the fact that the greater mass of yolk in the larger cell has displaced the cleavage plane to one side of its normal position.

Corresponding to this difference in the quantity of cytoplasm in the first two blastomeres of these eggs, there is a decided difference in the size of the nuclei and spheres, the latter always being proportional in size to the quantity of cytoplasm in which they lie. The smaller cells with the larger quantity of cytoplasm thus have larger nuclei and spheres than the larger cells, which have a smaller quantity of cytoplasm.

The eggs represented in figs. 13, 14, and 15 were centrifuged for five hours during the first cleavage and were then fixed at once. It is evident that division took place while the eggs were on the centrifugal machine and that the daughter nuclei have grown to the size shown while the eggs were still being centrifuged. Other eggs centrifuged for the same length of time were allowed to develop further after being removed from the centrifuge, and they show that in most cases the eggs were still alive after centrifuging and not seriously injured. Fig. 13 shows a very note-

worthy fact to which attention will be devoted in a future paper, viz., that the cell axis, which is marked by the line passing through the nucleus and sphere (and centrosome), remains unchanged after centrifuging. In the stage shown in fig. 13, the spheres lie between the nuclei and the polar bodies in normal eggs, and although the positions of cytoplasm and yolk, and of the first cleavage plane have been changed in this egg, this cell polarity remains unchanged.

Fig. 16 represents an egg which was centrifuged thirty minutes and then left in sea water for twenty hours. Neither this egg nor any others of this lot developed far after being centrifuged; it is possible that the eggs were injured in some way so that none of them developed, or it is barely possible that the record of the experiment is wrong. In all the eggs of this lot the appearance is that of eggs which had been under normal conditions for about three or four hours after being removed from the centrifuge.

This egg was evidently centrifuged during the first cleavage, which was very unequal, practically all of the cytoplasm having gone into the smaller of the two daughter cells. The nucleus and sphere in this smaller cell are enormous, whereas in the larger yolk cell they are extremely small, indeed no larger than in the anaphase stage of division. The chromosomes form a compact mass which stains deeply and contains no achromatic material. The sphere is small also but the fact that it holds its normal position with respect to the nucleus shows not only that the polarity of the cell remains unchanged, but also that the material of the sphere is different from the ordinary cytoplasm. In many cases similar to fig. 16 cytoplasm slowly forms around the chromosomes in the yolk cell and ultimately such a cell may develop in a normal manner. There is no evidence that cytoplasm ever passes through the cell membrane from one cell to another, and there is positive evidence that this does not occur. The formation of cytoplasm around a mass of chromosomes in a yolk field is therefore an occurrence of more than ordinary importance. The question has been asked frequently whether the nucleus alone can form cytoplasm or the cytoplasm alone a nucleus. It is known that the latter never happens; a mass of cytoplasm without

a nucleus may live for some time and show certain vital functions, but it is unable to grow or to regenerate lost parts. It is much more difficult to test the former question, for it is usually impossible to separate the nucleus completely from the cytoplasm and yet leave it in a medium in which growth would be possible. Verworn ('91) succeeded in shelling the nucleus out of *Thalassicola*, but found that the isolated nucleus was unable to grow a new cell body; but apart from the objection that the resting nucleus contains a large amount of cytoplasmic substance, this experiment is not conclusive for it is possible that the failure to grow a cell body was due to the lack of a proper nutrient medium in which the nucleus could operate.

The present experiment is free from most of these objections, though it must be confessed that one objection still remains, viz., it is not possible to be certain that every trace of cytoplasm has been removed from the yolk cell. Nevertheless the amount of cytoplasm left in the cell is very small and is quite indistinguishable, the only visible constituents of the cell being chromosomes, sphere and yolk. In the growth of cytoplasm in such a cell there first appears a very thin layer of cytoplasm around the chromosomes, then the yolk in the immediate periphery of this begins to dissolve and the cytoplasm increases in amount. Coincidentally the chromosomes swell up, absorbing achromatic material from the cytoplasm, and in later stages the growth of both cytoplasm and nucleus goes forward at an increasing rate. The formation of cytoplasm takes place only in the presence of chromatin and in its immediate vicinity; on the other hand the chromosomes grow only when surrounded by cytoplasm. This indicates that some influence, probably of a chemical nature, goes out from the chromosomes and leads to the solution of yolk and the formation of cytoplasm. Whether this influence from the chromosomes may act directly upon the yolk, or only indirectly through the medium of a minimal quantity of cytoplasm, is not certain, but it seems probable that the latter is the case. After cytoplasm has been formed around the chromosomes, but not before, the chromosomes themselves begin to swell up, absorbing achromatic material from the cytoplasm, and the chromatin grows in quantity. Cytó-

plasm is essential to the growth of the nucleus and of the chromatin; on the other hand chromatin is essential to the growth of cytoplasm, or to the conversion of yolk or food substances into cytoplasm. The life of the cell consists in an interchange of materials between the nucleus and the cytoplasm; the one cannot grow in the absence of the other. This conclusion agrees with the generalization of Godlewski ('10): "Zuerst das Bildingsmaterial geliefert und von den betreffenden Regeneratskomponenten zum Protoplasma assimiliert wird, dass dagegen in der zweiten Regenerationsphase dieses Protoplasma sich wenigstens teilweise zur Kernsubstanz transformiert" (p. 88).

The question has been much discussed as to whether the nuclei, and more particularly the chromosomes of the germ cells, are the sole 'bearers of heredity,' as Weismann, and many others, have maintained. We have experimental evidence that the cytoplasm cannot form chromatin in the absence of preëxistent chromatin. On the other hand there is no certain evidence that the chromatin can form cytoplasm in the absence of preëxisting cytoplasm. The experiment described above is not entirely conclusive, for while chromosomes in a yolk field form cytoplasm, it is probable that a minimal amount of cytoplasm is left in the yolk field, and it may be said that this merely grows by assimilation of yolk. On the other hand my experiments show that where we have equal division of the chromosomes and unequal division of the protoplasm we may have regulation and normal development; whereas this never follows abnormal distribution of the chromosomes; in other words protoplasmic abnormalities are capable of regulation when the nucleus is normal, but the reverse is not the case. The nucleus is the regulating center of the cell, and it is probably also the assimilating center. And since both of these functions are involved in inheritance, to this extent at least the nucleus may be said to be the inheritance center.

Fig. 17 represents an egg which was centrifuged for four hours during the first cleavage and was then placed under normal conditions for six hours before being killed. The polar body marks the original animal pole and in the centrifuging most of the yolk was thrown to this pole, most of the cytoplasm to the opposite



pole. The first cleavage plane is nearly equatorial in position, and one of the cells contains most of the cytoplasm. The spindles for the second cleavage have formed and the spindle in the cell containing the larger amount of cytoplasm is distinctly larger than the one in the other cell; each is proportional in size to the resting nucleus from which it came and to the volume of cytoplasm in the cell. The fact that the polarity of the cells has not been changed by the abnormal position of the first cleavage plane is indicated by the fact that the spindles are parallel to each other, but not to the plane of cleavage, as in normal eggs. In short there is evidence that the spindles here attempt to take up the positions which they would have occupied in a normal egg, with meridional cleavage.

Fig. 18 represents an egg from a lot which was centrifuged fifteen minutes in gum arabic, as recommended by Lyon ('04), and which was fixed three hours after removal from the centrifuge. Fig. 19 shows an egg which was centrifuged thirty minutes, and was fixed six hours later. In both cases the centrifuging took place during the first cleavage, as is shown by the unequal distribution of cytoplasm and yolk on both sides of the first cleavage plane. In the second cleavage, which evidently occurred after the eggs were removed from the centrifuge, the cytoplasm was distributed equally to the daughter cells. In fig. 18 the second cleavage took place a little earlier in the cell rich in cytoplasm (*AB*) than in the other (*CD*), but the smaller size of the nuclei in the latter is probably due in part to the fact that these cells are poor in cytoplasm. In fig. 19 the inequality in the distribution of cytoplasm at the first cleavage is much greater than in fig. 18; nevertheless the second cleavage occurred in the cell poor in cytoplasm (*CD*) at nearly the same time as in the other cell (*AB*). Although the nuclei in the cells *C* and *D* are much smaller than those in *A* and *B*, their structure shows that they are in nearly the same stage of the cell cycle. Their smaller size is due to the smaller quantity of cytoplasm in which they lie. Figs. 17-19 indicate that the absolute size of the nucleus has little to do with the time of its division; small nuclei in yolk-rich cells divide almost as rapidly as large nuclei in cells rich in cytoplasm.

Figs. 20 to 28 show eggs which were centrifuged during the second cleavage. The first and second cleavages may always be distinguished by the fact that the polar furrow bends to the right in the first cleavage and to the left in the second (Conklin '97). In fig. 20 the distribution of cytoplasm and yolk to the daughter cells was equal in the first cleavage but unequal in the second, and the daughter nuclei are proportional in size to the volume of the cytoplasm in which they lie.

In fig. 21, which represents an egg which was centrifuged for 30 minutes and fixed at once, the second cleavage is very unequal, two of the macromeres (*B* and *C*) being small protoplasmic cells, which resemble micromeres in appearance, but which behave like macromeres as the study of later stages (figs. 24 to 28) shows.

Fig. 22 represents an egg which was centrifuged for thirty minutes during the second cleavage and then kept under normal conditions for twenty-one hours before being fixed. The second cleavage was suppressed although the nucleus divided in the upper cell, *AB*, but not completely in the lower one, *CD*. These nuclei have given rise to spindles for the third cleavage, there being two independent spindles in the cell *AB*, and two spindles which are fused at one pole in the cell *CD*, thus forming a triaster. The degree of abnormality in this case is indicated by the fact that the development has been halted at this stage, although a normal egg would have reached the 20-cell stage at least, in the time which elapsed after centrifuging.

With the exception of fig. 26, all of the figs. from 23 to 28 were drawn from the same lot of eggs which were centrifuged for thirty minutes in the 2-cell stage, and then kept for six hours under normal conditions before being fixed. In all of these eggs the second cleavage was made very unequal by the centrifuging. Two of the macromeres are not only much smaller than the other two, but are composed entirely of cytoplasm, whereas the two larger macromeres contain all of the yolk. Nevertheless the behavior of these two small, protoplasmic macromeres is almost identically like that of the large, yolk-rich macromeres; the micromeres are given off from both the protoplasmic and the yolk laden macromeres at practically the same time and in the same

direction; the micromeres formed from these abnormal macromeres are the same as in normal eggs in which all the macromeres are of the same size and contain the same quantity of cytoplasm and yolk. In short there is here a form of regulation which leads to the formation of normal micromeres from abnormal macromeres, and the exact manner in which this cellular regulation takes place is of fundamental importance, and will be discussed later.

Fig. 23 represents an egg similar in many respects to fig. 21, but of a later stage. The smaller protoplasmic macromeres preserve their original polarity as is shown by the fact that the spheres lie between the nuclei and the polar bodies. On the other hand each of the large macromeres contains a tetraster; the spindles are those of the third cleavage.

Fig. 24 represents an egg of the same type as the preceding, after the third cleavage; each macromere has given rise to a micromere which is normal in form, position, constitution and size, although the macromeres are very abnormal in these regards, two of them containing all of the yolk and very little protoplasm, and the other two being small and purely protoplasmic. Indeed the macromeres *1A* and *1D* nearly exhausted all the cytoplasm which they contained in order to form cytoplasmic micromeres of normal size; on the other hand, the size of the micromeres *1b* and *1c* is not influenced by the fact that the macromeres from which they come are small and are purely protoplasmic.

Fig. 25 is a drawing of an egg in a slightly older stage than fig. 24; the large macromeres, *1B* and *1C*, are giving off the second set of micromeres, *2b* and *2c*, while two of the first set of micromeres, *1b* and *1c*, are just beginning to divide. The four small cells which lie to the left of the polar bodies are the macromeres *1A* and *1D* and the micromeres *1a* and *1d*; these cells are purely protoplasmic and are very small, all four of them being no larger than one of the micromeres, *1b* or *1c*, in the other quadrants. Nevertheless these minute 'macromeres' have each given rise by an equal cleavage, to a micromere as large as itself. Although these micromeres are much smaller than those in the other quadrants, they are the largest that could be formed from the macro-

meres in question without making the macromeres smaller than the micromeres, thus reversing the usual inequality of this division; in short the division of these cells represents the nearest possible approach to normal conditions.

Figs. 27 and 28 show eggs of the same type as the preceding, but at a stage after the formation of the second set of micromeres (*2a-2d*) and during the division of the first set (*1a-1d*). Here also these micromeres are normal in size, although the size relations, and the cytoplasmic or yolk content of the macromeres from which they came, are very abnormal. In the cleavages which follow after the centrifuging, complete regulation has occurred, so far as this is possible. It is not possible for regulation to take place by the redistribution of cytoplasm and yolk by passage through a cell membrane.

Fig. 26 represents an egg which was centrifuged for thirty minutes during the second cleavage, and then fixed twelve hours later. At the time of centrifuging the nuclear division in the second cleavage was complete, but the division of the cell body was suppressed. Consequently each of the blastomeres, *AB* and *CD*, contained two nuclei, which by subsequent division in the manner indicated in fig. 22 have given rise to two sets of micromeres, *1a-1d*, and *2a-2d*. Both sets of micromeres have divided, as indicated by the connecting bonds, thus forming a somewhat abnormal cap of sixteen micromeres. The nuclei of the macromeres are indicated by the reference lines from the letters *2A-2D*. Other cases similar to this one will be shown and described in another paper, but this one egg shows that it is possible for both the nuclei of a binucleate cell to divide at the same time and to give rise to separate cells, each with a single nucleus, and that such cells may approximate in form and position normal blastomeres.

Fig. 29 represents an egg which was centrifuged for four hours at the close of the second cleavage, and fixed at once after centrifuging. The yolk has been forced out into lobes, which are still connected with the protoplasmic portions of the cells except in the case of one cell, where the lobe has been completely separated. It is a significant fact that the point at which the lobe forms, and consequently the point where the cell membrane is weakest lies

at the outer pole of the axis which passes through the centrosome and nucleus and these axes mark the position of the spindles of the third cleavage. Here, as in every other instance, the smallest nucleus is found in the cell which has the smallest amount of cytoplasm.

Fig. 30 is a drawing of an egg which was centrifuged ten minutes in gum arabic, during the first cleavage, and fixed four hours later during the third cleavage. Macromeres *A* and *B* are richer in cytoplasm and poorer in yolk than *C* and *D*, and correspondingly the spindles and asters are larger in the former than in the latter.

Figs. 31 and 32 represent eggs which were centrifuged four hours during the first cleavage, and were fixed six hours later. In both eggs the macromeres *A* and *B* are richer in cytoplasm and poorer in yolk than *C* and *D*. In fig. 31 the cells *A* and *B* contained more cytoplasm and divided earlier than *C* and *D*; at least one-half of the cytoplasm in the latter cells has gone into the formation of the micromeres, which are still, however, smaller than normal. The first cleavage in this egg did not pass through the animal pole, marked by the polar bodies, but was displaced to one side, and the spiral form of the cleavage is not clearly preserved in the cells *C* and *D*. While the regulation in the size of these micromeres is not complete, the tendency to approach the normal condition is evident. Fig. 32 is similar to fig. 31, though the macromeres *C* and *D* of this egg contained a larger amount of cytoplasm than in fig. 31, and the regulation in the size of the micromeres is complete.

Fig. 33 shows an egg, from the same slide as fig. 30, which was centrifuged ten minutes in gum arabic and fixed four hours later. The macromeres *1A* and *1B* contain more cytoplasm and are dividing earlier than *1C* and *1D*, but the micromeres from the former are no larger than those from the latter.

Fig. 34 represents an egg which was centrifuged for two and one-half hours during the first cleavage, and was fixed twenty-one hours later. The macromeres *2A* and *2B* contain much cytoplasm, while *2C* and *2D* contain little and yet the micromeres formed from the latter are almost as large as those from the former.

Figs. 35, 36, 37 represent eggs, from the same experiment, which were centrifuged thirty minutes during the first cleavage, and were fixed twelve hours later. The size of the micromeres of the first, second or third sets is but little influenced by the quantity of cytoplasm in the macromeres; the size regulation of the micromeres is here practically complete. In fig. 37 the cell 4c forms at the same time as 4d, though in normal eggs it does not form until much later; the precocious formation of this cell is probably due to the fact that the amount of cytoplasm in macromere C was larger than normal.

### *III. General results of these experiments*

The results of these experiments, which have been described in the order of development from the earlier to the later stages without reference to a logical presentation of general questions, may now be classified and compared with the observations on cell size and nuclear size given in Part I of this paper. In general these experiments support in every detail the conclusions based upon the study of normal eggs and blastomeres.

1. *Nuclear size in centrifuged eggs.* In centrifuged eggs, as in normal ones, the size of the nucleus is always dependent upon the quantity of cytoplasm surrounding the nucleus and upon the length of the resting period. Nuclei which are normally large may be caused to remain small, and nuclei which are normally small may be rendered large by merely changing the positions of the yolk and cytoplasm in the cell.

In normal eggs of *Crepidula* the egg nucleus lies in a protoplasmic field near the animal pole of the egg, while the sperm nucleus enters the egg near the vegetal pole and moves up toward the animal pole through a field of yolk. As long as the sperm nucleus is in this yolk it remains very small, and only when it emerges into the protoplasmic field near the egg nucleus does it begin to grow rapidly. The egg nucleus on the other hand, grows rapidly and becomes much larger than the sperm nucleus. If now an egg is centrifuged during the formation of the second polar body so as to throw the yolk to the animal pole and the cytoplasm to

the vegetal pole, the normal size relations of the germ nuclei is reversed, the sperm nucleus becoming larger than the egg nucleus as shown in fig. 11. Godlewski ('08) holds that the size of the sperm nucleus depends upon the time which elapses before its union with the egg nucleus; it also depends, as I have shown, upon the quantity of cytoplasm in which it lies. We conclude therefore, that in all animals the relative sizes of egg and sperm nuclei are dependent upon the amount of cytoplasm in which they lie, and upon the length of the growth period (interkinesis). In this connection it may be worth while to remark that one reason why the rhythm of cleavage, in Boveri's, Driesch's, and Godlewski's experiments, follows the maternal rather than the paternal type may be found in the fact that the rate of growth of the nucleus is dependent upon the quantity and quality of the protoplasm of the egg.

In the cleavage of the egg the size of the nucleus is dependent upon the quantity of protoplasm in which it lies, as shown by figs. 12 to 20. In eggs subjected to strong centrifugal force the egg contents separate into three zones, a yellow zone of yolk at the distal (heavy) pole, a gray zone of oily and watery substance at the central (light) pole, and a clear zone of protoplasm between these two. It is the latter substance which contributes to the growth of the nucleus, as is shown by such cases as fig. 16 in which the gray substance was centrifuged out of the egg and practically all of the yolk thrown into one of the blastomeres, and most of the clear protoplasm into the other; the nucleus in the blastomere which contains yolk but little or no protoplasm has scarcely grown at all, the one in the cell containing the clear protoplasm, but without the gray substance, has grown enormously. Similar, though less striking, differences in the sizes of nuclei, depending upon the quantity of clear protoplasm in the cell, are found in all the eggs figured. In centrifuged eggs the nucleus always occupies the middle zone, and as I have just shown it grows at the expense of substance received directly from this zone. The fact that the specific gravity of the nucleus and of this middle zone are the same, is probably due to the fact that so much of the absorbed nuclear material is from this zone.

2. *The sizes of spindles, centrosomes, spheres and asters.* The study of centrifuged eggs shows, as was observed in the case of normal eggs, that the sizes of spindles, centrosomes, spheres and asters are dependent upon the quantity of cytoplasm in which they lie. The size of the spindle is also related to the size of the nucleus, as I have already shown, but as this, in turn, is dependent upon the quantity of cytoplasm of the middle zone, it follows that the size of the spindle as well as that of the centrosome and sphere is related to the quantity of cytoplasm in which they lie. Fig. 17 shows spindles in sister cells which are quite different in size owing to the different amounts of cytoplasm in these two cells; while figs. 11, 12, and 16 show centrosomes and sphere which vary in size depending upon the quantity of cytoplasm surrounding them.

In this connection attention should be called to the fact that the spindles from the stage of the metaphase to the end of mitosis are anchored in the cell, and can be moved only with much difficulty. The spindle fibers are tougher and more consistent than the surrounding plasma, and they are not a mere arrangement of granules in the lines of force as Lillie ('09) has maintained for *Chaetopterus*.

3. *The rhythm of division in centrifuged eggs.* The rhythm of division is not dependent solely upon nuclear size, nor cell size, nor the ratio of one to the other (Kernplasma-Relation), though it may be influenced by the absolute amount of cytoplasm present in the cell. Cleavage cells which contain a large amount of cytoplasm, and which therefore have large nuclei, usually divide a little earlier than cells poor in cytoplasm, and with small nuclei, though this is not always the case, as is shown by fig. 17, in which the large and the small nuclei divide at the same time. Nuclei which differ greatly in size may still be in the same stage of the nuclear cycle, as shown in fig. 19, and may divide at the same time. On the other hand, figs. 25, 31, 34 and 37 show cases in which nuclei of the same generation divide earlier in cells rich in cytoplasm than in cells which are poor in this substance.

4. *Growth of cytoplasm at the expense of yolk.* Centrifuged eggs afford an excellent opportunity of studying the way in which



cytoplasm grows at the expense of yolk. In cases in which the centrifuging occurred after the spindle was anchored in the cell, but before the division wall had formed, the cytoplasm may be thrown almost entirely to one pole of the spindle and the yolk to the other; accordingly when division occurs one of the daughter cells will contain almost all the cytoplasm, the other all the yolk, while both cells will receive the same number and mass of chromosomes, fig. 16. The chromosomes which are left in the yolk field remain small and compact since there is no cell substance which they can absorb. After some time the yolk in the vicinity of the chromosomes may begin to disappear and cytoplasm to appear in its place. It can scarcely be doubted that some substance, probably an enzyme, is given off by the chromosomes and dissolves the yolk, and that this dissolved yolk is then converted into cytoplasm through the influence of the chromosomes. Once a small field of cytoplasm is formed around the chromosomes, they begin to absorb it and to become vesicular. The process of forming cytoplasm may then go forward rapidly and in the end the yolk cell may give rise to protoplasmic micromeres in a normal manner (fig. 31). It is probable that a small amount of cytoplasm, which cannot be displaced by centrifuging, is left in the yolk cell, and it is possible that the formation of new cytoplasm would not take place in the absence of this small remnant, but it can be proved conclusively that this formation of cytoplasm takes place only in the vicinity of the chromosomes, and that in the absence of this chromatic material it never occurs at all. Under these circumstances the conclusion seems justified that the chromatin has the power of forming cytoplasm when placed in a suitable nutrient medium, such as yolk, and that the cytoplasm in turn contributes to the growth of the nucleus and of the chromatin.

5. *Unequal and differential cell divisions.* By centrifuging, the size and constitution of the blastomeres may be changed; divisions which are normally equal may be made unequal, and *vice versa*; cells which are normally protoplasmic may be filled with yolk and *vice versa*. In this way both the cell size and the cell content may be controlled experimentally.

Acknowledgedly the position of the spindle conditions the plane of the cleavage, the division wall passing through the equator of the spindle. When by any means the spindle is displaced from its normal position the division plane is displaced. In this way giant polar bodies may be formed, as shown in fig. 11, or macromeres may be formed which are small and free from yolk, as shown in figs. 16, 21-28, *et al.*

Are the inequalities and differentiations of normal cleavage due to similar causes, viz., external or internal pressure? Clearly external pressure cannot be involved in the unequal division of free cells, such as the maturation divisions of the egg; and the fact that isolated blastomeres of the 4-cell stage divide in the normal manner into small protoplasmic micromeres and large yolk-rich macromeres, shows that these unequal divisions during the cleavage period cannot be explained as the result of reciprocal pressure among cells. On the other hand, the formation of micromeres of normal size and constitution from purely protoplasmic macromeres, as shown in figs. 24, 27, 28, 33, 36, *et al.*, indicates that this inequality of division cannot be due to the crowding of the spindle to one side of the cell by internal pressure, such as might come from the presence of a mass of yolk—because in the cases cited, little or no yolk is present in the macromeres. If internal pressure is involved in the unequal division of these protoplasmic cells it must be pressure of a very different sort from that involved in the presence of a mass of metabolic products at one side of the cell. While the spindle may be pressed out of position by external or internal pressure this will not serve to explain the eccentric position of the spindle in such cases as I have described.

A satisfactory explanation of unequal and differential cell division must also be able to be applied to equal and non-differential cleavage, for the causes of the latter are not simple mechanical conditions, such as pressure. In the case of cleavages which are normally equal, if the spindle and yolk are moved to eccentric positions in the cell, they come back, if possible, to their normal positions when the pressure is removed; indeed they sometimes seem to come back against considerable pressure, as when

a spindle moves out of a protoplasmic field into the yolk in order to reach its normal position in the cell. When eggs like the one shown in fig. 11 are removed from the centrifuge, the egg and sperm nuclei, together with the cytoplasm surrounding them move up through the yolk until they ultimately lie in their normal position on the animal side of the egg, beneath the polar bodies. However far the germ nuclei or the first cleavage spindle may be removed from the chief axis of the egg, they invariably come back to their normal positions, with the equator of the spindle in the egg axis, and the long axis of the spindle at right angles to the egg axis, unless the spindle is held so long in its abnormal position that it is caught in that position by the divisional processes. The same is true also of the nuclei and spindles of the 2-cell stage; when moved out of the median plane of the cell they come back to that median plane, unless the cells are injured or the spindles are held in their abnormal position until the metaphase or a little later. Evidently the cause of equal cell division, such as the first and second cleavages of *Crepidula*, is not so simple as those have assumed who have attributed it to pressure, the line of least resistance, or the long axis of the protoplasmic mass.

Not only the eccentricity or lack of eccentricity, but also the axis of the spindle is of great importance in determining the character of the cleavage. While the former is associated with the equality or inequality of division, the latter conditions its differential or non-differential character. The polar differentiation of the egg is the first visible morphogenetic differentiation, and it is not without significance that in the first and second cleavages of the egg the spindles are at right angles to the egg axis, while in the third, fourth and fifth cleavages they are more nearly parallel with that axis.

I have hitherto spoken of the position of the spindle as if it were the one cause of equal or unequal, differential or non-differential cleavage; but for many reasons it is evident that the position of the spindle is itself the result of the structure or organization of the protoplasm, and that in this organization polarity and symmetry play an important part. Many years ago ('93) I showed that even before the spindle is formed, the shape of the

cell may indicate the position and direction of the coming cleavage, and I maintained then and in subsequent papers ('97, '99, '02) that the position of the spindle and the size, position, and histological character of the daughter cells is the result of the structure of the protoplasm, and particularly of the polarity and symmetry of the cell.

These conclusions have been confirmed by much experimental work on cell division, which I have completed but have not yet published. The position of the spindle and the plane of cleavage may be greatly changed, but the polarity and organization of the protoplasm remain unchanged, as I shall show in a future paper. Indeed it is very difficult to alter the polarity of any cell as Lillie ('06, '09) has shown, and one reason for this is to be found in the fact, as I have discovered in *Crepidula*, that the cell axis, i.e., the axis connecting nucleus and centrosome, can rarely be changed by artificial means.

6. *Regulation in the cleavage process.* Evidently connected with this persistent organization of the cell is the power of regulation which is shown in the cleavage of the egg as well as in the regeneration of adult parts. Whenever the size or constitution of blastomeres of *Crepidula* have been changed, or when cleavages have been suppressed, subsequent cleavages come back to the normal form so far as this is possible. The original disturbance can be righted only very gradually if at all, since neither yolk, cytoplasm nor nuclei can pass through cell membranes, and the only redistribution of substances possible is by means of new cell divisions. But in *Crepidula* the divisions following upon such a disturbance of the usual cleavage process are almost if not entirely normal. This is very evident in the divisions following upon disturbances of the first two cleavages. All of the yolk may be centrifuged into two of the macromeres and practically all of the cytoplasm into the other two, as in figs. 16, 19, 21, 23, *et. al.*; two of the 'macromeres' may be very small and two very large, as in figs. 16, 21, 23 to 28; or one of these first two cleavages may be suppressed, as in figs. 22 and 26; but if such abnormal eggs are allowed to develop under normal conditions, the micromeres are formed in normal manner, as is shown in figs. 24 to 28 and 32

to 37. Whatever the content of the different macromeres may be, whether purely protoplasmic or entirely yolk the micromeres are always protoplasmic, even though division must be delayed until the cytoplasm which goes into the micromeres can be formed from yolk (figs. 24, 31, 35); whatever the size of the macromeres, the micromeres formed from them are approximately normal in size, even though yolk-rich cells must give up most of their cytoplasm (figs. 24, 31, 35), or protoplasmic micromeres must divide equally (figs. 25, 28), in order to give rise to micromeres of the usual size.

Such regulations of cleavage are probably caused, in the case of *Crepidula*, by the persistent polarity of each cell, which in turn leads to the localization of the spindle in a definite axis, with its pole at a definite distance from the surface of the cell. In what manner the polarity of the cell may cause the localization of the spindle is clearly shown in the cleavage of *Crepidula*. In former publications ('99, '02) I have called attention to the fact that definite movements of cell substance take place in dividing cells, and that these movements serve to orient the spindles; these movements are always related to the polarity of the cell and to that of the entire egg. Furthermore, I have elsewhere ('02) called attention to the fact that the cell membrane is weakest opposite the poles of the spindle. I was formerly of the opinion that this was due to some influence of the spindle on the cell membrane, but a further study shows that these weak places in the cell membrane are present before the spindle forms and can not therefore be caused by the spindle. In the egg shown in fig. 29 the places of reduced tension on the cell membrane are indicated by the lobes of yolk attached to the cells, and a line drawn through the centrosome, nucleus and lobe indicates the precise position which the spindle will take at the next cleavage. The axes of the third cleavage spindles are here marked out long before the spindles are formed; the weak spot in the cell membrane is not caused by the position of the spindle, but the latter is the result of the former. Experiments on eggs in the 2-cell, 4-cell and 8-cell stages of cleavage show that the positions of the points where the membrane is weakest, change in each cell generation and that they

always mark out the position of the spindle. These lobes are formed only when the egg is subjected to pressure and then only at those points on the cell surface which mark the position which will be taken by the poles of the spindle. Since the spindle axes change in successive cleavages it follows that this point of reduced tension also changes in successive cell generations.

I conclude therefore that the position of the spindle, and all the morphogenetic results which follow from this, is dependent upon the polarity of the cell; which polarity manifests itself not only in the localization of cytoplasmic substances, but also, and more fundamentally, in definite movements of the oöplasm and in reduced tension of the cell membrane at the poles of the cell.

#### GENERAL SUMMARY AND INDEX

##### *Part I. Observations*

1. The equality or inequality of cell division in normal cleavage is due to internal causes, rather than to the presence of metabolic substances, such as yolk, within the cell or to pressure from without. These internal causes are to be found in the polarity of the cell, in movements of the cytoplasm, and in the structure of the cell membrane. Since the position and axes of the spindles change regularly in successive divisions this protoplasmic organization must also change regularly (pp. 6-9).

2. The yolk-lobe is a temporary extrusion of yolk or oöplasm during mitotic pressure, at the former point of attachment to the ovarian wall and a little to one side of the vegetative pole. If this lobe is large, the resulting cleavage is unequal, although the furrow cuts through the chief axis and the center of the egg. The degree of inequality of the first and second cleavages is measured by the size of the yolk-lobe. The yolk-lobe is the result of an unsymmetrical distribution of yolk or egg substance with reference to the egg axis (pp. 9-11).

3. In *Crepidula plana* the Kernplasma-Relation varies greatly in different blastomeres and at different stages, depending chiefly upon the length of the resting period (interkinesis). In cases

where nuclei and cells are measured at their maximum size it varies from 14.5 to 0.37; at mean size from 35.7 to 1.1; at minimum nuclear and cell size it varies from 285 to 29. In protoplasmic blastomeres, which contain no yolk, the Kernplasma-Relation varies from 14.5 to 8.7, when the nuclei are at their maximum size; and from 35.7 to 7, when the nuclei are at mean size. In Fulgur, at mean size, it varies from 127.7 to 3.6 (pp. 16-24).

4. In different eggs, corresponding blastomeres have approximately the same Kernplasma-Relation; but in different blastomeres of the same egg or of different eggs the Kernplasma-Relation is neither a constant nor a self regulating ratio. It appears to be a result rather than a cause of the rate of cell division, and consequently a variable rather than a constant factor (pp. 24-25).

5. In the tissue cells of adult *Crepidulas* there is no marked increase of cytoplasm over nucleus, as compared with the blastomeres. The Kernplasma-Relation of various adult epithelial cells, not filled with metabolic products, varies from 28 to 7; in oöcytes and ganglion cells it varies from 6 to 3 (pp. 25-28).

6. The size of the nucleus is dependent upon at least three factors: (a) The initial quantity of chromatin (Boveri); (b) The volume of the cytoplasm; (c) The length of the resting period (p. 25).

7. The inciting cause of cell division in *Crepidula* is not found solely in the limitations of the working sphere of the nucleus (Strasburger), nor in the doubling of the volume of the chromosomes (Boveri), nor in a Kernplasma-Spannung (Hertwig), but rather in the coincidence of centrosomal, chromosomal and cytoplasmic rhythms, which are probably connected with the rate and nature of metabolism in the cell (pp. 29-32).

8. During the cleavage of the egg of *Crepidula plana* the volume of the cytoplasm more than doubles between the 1-cell and the 24-cell stage the average growth for each division being about 6 per cent; the yolk decreases in volume by nearly one-half and the entire egg is smaller at the 24-cell stage than at the 1-cell stage. This can only mean that the yolk contributes to the growth of cytoplasm during the cleavage period (pp. 32-36).

9. The average nuclear growth during cleavage is not more than 5 per cent to 9 per cent for each division up to the 32-cell stage and it may fall as low as 0.3 per cent to 1 per cent for each division after that stage; and in every case it falls far short of a doubling, or increase of 100 per cent, for each division (pp. 36-44, 54, 55).

10. Both nuclear sap and linin belong to the cytoplasm as well as to the nucleus. The chromatin is the most distinctive nuclear substance. All of these constituents are more abundant in large cells than in small ones. The mitotic spindle is of both nuclear and cytoplasmic origin and its size depends upon the volume of both nucleus and cytoplasm (pp. 44-47, 55).

11. The average growth in volume of chromatin from the 2-cell to the 32-cell stage is about 8 per cent for each division period, being about the same as the growth of the nucleus as a whole (pp. 47-48, 55).

12. The chromosomes become individually smaller as cleavage progresses, and in general small nuclei give rise to smaller chromosomes than do large nuclei (pp. 48-51, 55).

13. The size of the nucleoli (plasmasomes) depends upon the size of the nucleus and the length of the resting period; the larger the nucleus and the longer the resting period, the larger the plasmasomes become (pp. 51-53, 55-56).

14. Centrosomes and spheres of large cells are larger than those of smaller ones (pp. 53, 56).

15. The rate of growth of chromatin during the early cleavages of *Crepidula* (8 per cent for each division) harmonizing with the slight rate of increase of the oxidative power of the egg as determined by Warburg (p. 56).

16. My observations do not support the view that senescence is due to a decrease (Minot), or an increase (Hertwig) of nuclear, as compared with protoplasmic material; nor that rejuvenescence is accomplished during cleavage by the great increase of nuclear material relative to the protoplasm. On the other hand senescence seems to be associated with a decrease, rejuvenescence with an increase of metabolism (Child). Anything which decreases the interchange between nucleus and cytoplasm, such as products



of differentiation and metabolism within the cell, or a dense nuclear membrane, decreases metabolism and leads to senescence; anything which facilitates this interchange increases metabolism and leads to rejuvenescence. It is suggestive that in early development increased oxidation is associated with fertilization and mitosis (Loeb, Lyon, Warburg) (pp. 57-62).

### *Part II. Experiments*

17. By centrifugal force the substance of the eggs and blastomeres of *Crepidula* may be stratified into a zone of heavy yolk at one pole, a zone of lighter oil and water at the other pole, and a zone of clear cytoplasm between these two; and since these eggs orient but slightly if at all while being centrifuged, the axis of centrifuging and of stratification may form any angle with the egg axis. In the early development of *Crepidula* the volume of yolk is much greater than the volume of cytoplasm and consequently the latter may be displaced to any side of the center of the egg or blastomere (p. 64).

18. On the other hand the mitotic figure, after the prophase, can be moved only with great difficulty, and owing to this fact the substances of a cell can be distributed in very atypical manner with respect to the poles of the spindle and the resulting daughter cells. In this way all the yolk present in a dividing cell may be thrown into one of the daughter cells, and almost all of the cytoplasm into the other (p. 64).

19. These experiments show that the spindle is a specific structure and not merely a dynamic expression of lines of force. It remains in position and functions normally when the substance in which it usually lies is completely replaced by other substance. The spindle fibers are denser than the general cytoplasm and may be stretched, shortened or bent by pressure (p. 65).

20. If centrifuging occurs during the second maturation division, when the poles of the egg are clearly marked, the yolk may be driven to the animal pole and the cytoplasm to the vegetal pole, the spindle may be much elongated and a giant polar body may be formed (fig. 11). In such cases the sperm nucleus, which

enters the egg near the vegetal pole, lies in a cytoplasmic field, the egg nucleus in a yolk field, and the former grows more rapidly than the latter, thus reversing the usual size relations of the germ nuclei. The relative size of the germ nuclei is dependent upon the volume of the cytoplasm in which they lie as well as upon the length of time that the sperm nucleus has been in the egg (pp. 67, 75).

21. If centrifuging occurs during the cleavage almost all the yolk present may go into one daughter cell, almost all the cytoplasm into the other (figs. 16, 19). Under these circumstances the subsequent growth of the daughter nuclei is proportional to the volume of the cytoplasm of the middle zone in which they lie. Neither the yolk nor the substances of the lighter zone contribute directly to the growth of the nucleus (pp. 75-76).

22. The size of spindle, centrosome, and sphere in any cell is not definitely fixed, but may be modified by altering the quantity of cytoplasm; the larger the quantity of cytoplasm in a cell, the larger are all the structures named (p. 77).

23. The rhythm of division may be modified, but only to a slight extent, by altering the quantity of cytoplasm in a cell. In general, cells rich in cytoplasm divide a little earlier than those poor in this substance; but though the quantity of cytoplasm in a cell and the size of its nucleus may be greatly changed by centrifuging, the rhythm of cleavage is but slightly changed (p. 77).

24. When the daughter chromosomes at one pole of a spindle are left in a cell composed almost entirely of yolk, they do not form a vesicular nucleus until yolk has been dissolved and a certain amount of cytoplasm has been formed around the chromosomes. It is evident that something, perhaps an enzyme, is given off from the chromosomes or chromatin, which leads to the transformation of yolk into cytoplasm; this cytoplasm is in turn taken up by the chromosomes and ultimately contributes to the growth of the chromatin, (pp. 77-78).

25. The typical size, position and constitution of blastomeres, and consequently the type of cleavage, do not depend upon external or internal pressure, but upon a definite polarity, symmetry and movement of the cell contents, and upon reduced surface

tension at the poles of the cell. Therefore, the causes of equal or unequal, differential or non-differential divisions are intrinsic rather than extrinsic (pp. 78-81).

26. Whenever the size, constitution or number of blastomeres is changed from the typical condition, subsequent cleavages come back to the normal form so far as this is possible. This regulation in cleavage is connected with a persistent polarity of the cell, which is not changed by centrifuging, and which manifests itself in a definite cell axis passing through nucleus and centrosome, in typical movements and localizations of cell contents, and in reduced tension of cell membrane at the poles of the cell (pp. 81-83).

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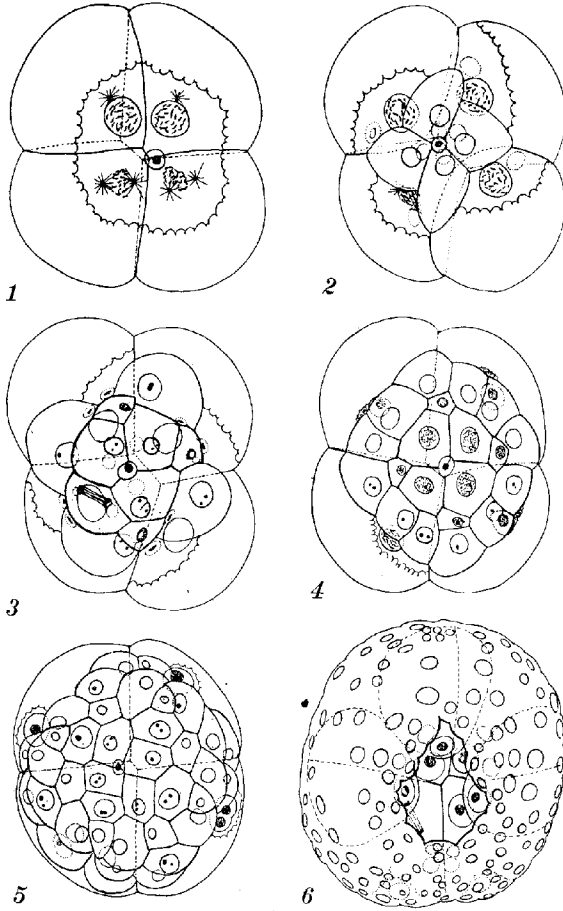
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## DESCRIPTION OF FIGURES

\*All figures (with the exception of figs. 9 and 10) represent entire eggs of *Crepidula plana*, fixed, stained, and mounted on slides. They were drawn with the aid of a camera lucida under Zeiss Apochromat 3 mm., Öcular 4, and represent a magnification of 333 diameters. In the centrifuged eggs, the axis of centrifuging is, in many cases, indicated by an arrow, the head of the arrow marking the distal (heavy) pole and the tail of the arrow the central (light) pole. In figs. 12 to 19, and 29 to 37 the first cleavage is in the long axis of the page, the second cleavage (figs. 18 and 19) is at right angles to this. In figs. 21 to 28 the first cleavage runs across the page, the second, lengthwise of it.



Figs. 1-6 Successive stages in the development of the egg of *C. plana*, showing the maximum sizes of the nuclei of the macromeres. Fig. 1, 4-cell, just before third cleavage; fig. 2, 8-cell, just before fourth cleavage; fig. 3, 16-cell, just before fifth cleavage; fig. 4, 24-cell, just before sixth cleavage in macromere 3D; fig. 5, 42-cell, just before sixth cleavage in macromeres 3A-3C; fig. 6, Gastrula, just before seventh cleavage of the macromeres.

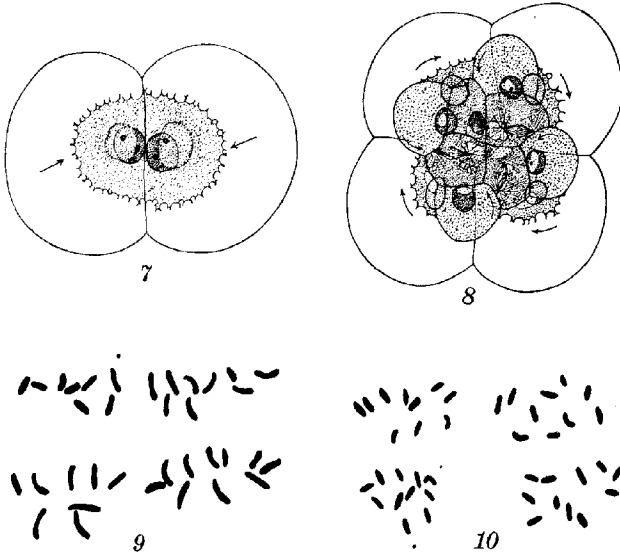


Fig. 7 2-cell stage of *C. plana*. The nuclei just before the second cleavage are  $24\mu$  in diameter.

Fig. 8 12-cell stage of *C. plana*. The nuclei in the first quartet of micromeres, 1a-1d, three of which are dividing, are  $14\mu$  in diameter at their maximum size.

Fig. 9 Chromosomes from four different spindles of the second cleavage, all in the metaphase and all magnified 2000 diameters.

Fig. 10 Chromosomes from four different spindles of the cells 1a-1d, all in the metaphase and all magnified 2000 diameters.

Fig. 11 Egg centrifuged ten minutes after formation of first polar body and during formation of second; fixed three hours after centrifuging. Telophase of second maturation division; indication of formation of enormous second polar body. The size of nuclei is dependent upon quantity of cytoplasm in which they lie.

Fig. 12 Centrifuged fifteen minutes in gum arabic, fixed three hours later. Evidently centrifuged during first cleavage; almost all of the cytoplasm is in the smaller cell. The size of the nuclei is proportional to the quantity of cytoplasm.

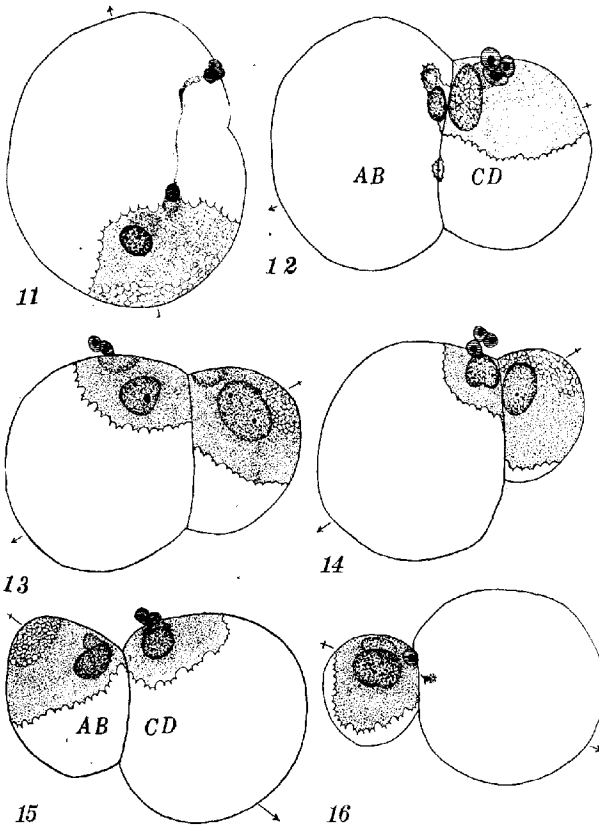


Fig. 13 Centrifuged five hours (2000 revolutions per minute) during the first cleavage; fixed at once; structure similar to preceding.

Fig. 14 From the same experiment as the preceding. Size of nuclei is proportional to the quantity of clear (granular) cytoplasm; yolk and oily or watery constituents of the cytoplasm do not influence nuclear size.

Fig. 15 From the same experiment as the preceding, and showing similar results.

Fig. 16 Centrifuged thirty minutes; fixed twenty hours after centrifuging. Egg has not developed. Enormous difference in the size of sister nuclei.



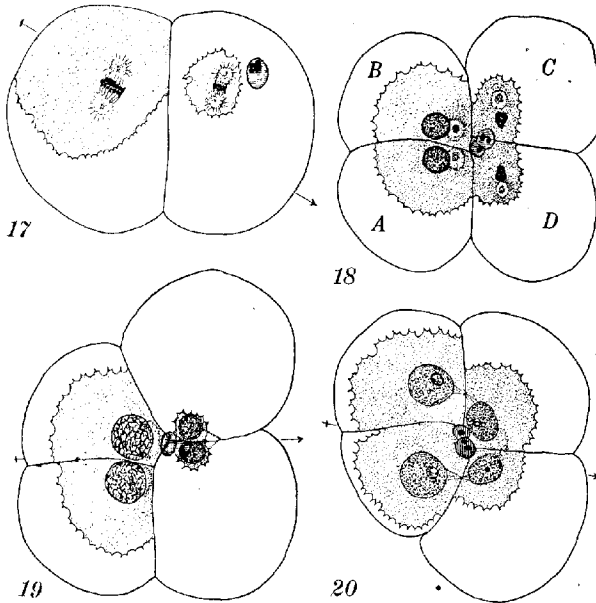


Fig. 17 Centrifuged four hours (2000 revolutions per minute); fixed six hours after. Evidently centrifuged during first cleavage. The cleavage plane does not pass through the polar axis. The spindles are proportional to the size of nuclei from which they were formed, and to the volume of cytoplasm in which they lie. They are not parallel to the plane of the first cleavage, which is here out of its normal position.

Fig. 18 Centrifuged fifteen minutes in gum arabic; fixed three hours after. Evidently centrifuged during first cleavage. The second cleavage appeared earlier in the more protoplasmic cells (A and B), than in the others.

Fig. 19 Centrifuged thirty minutes; fixed six hours later. Evidently centrifuged during the first cleavage. The size of the nuclei is plainly dependent upon the volume of the cytoplasm in which they lie.

Fig. 20 Centrifuged four hours, (2000 revolutions per minute); fixed at once. Evidently centrifuged during the second cleavage; the daughter nuclei are proportional in size to the volume of cytoplasm in which they lie.

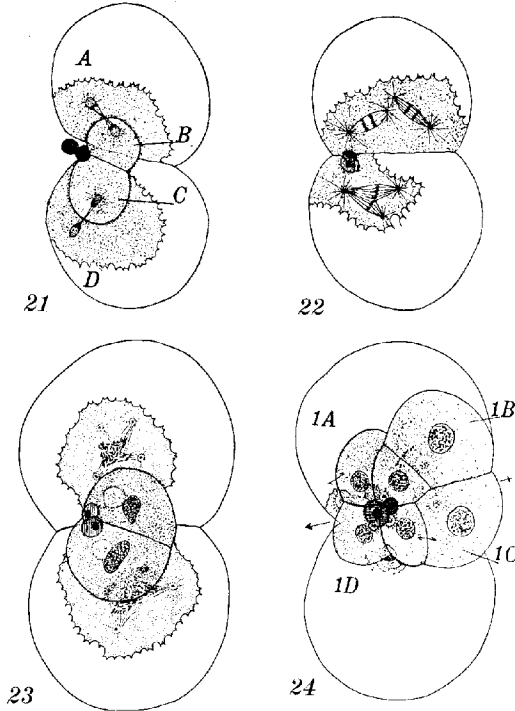


Fig. 21 Centrifuged thirty minutes; fixed at once. Centrifuged during the second cleavage, which was thus made very unequal, two of the macromeres (A and D) containing all the yolk and the other two (B and C) being small and purely protoplasmic.

Fig. 22 Centrifuged thirty minutes; fixed twenty-one hours later. The second cleavage was suppressed. Two spindles for the third cleavage are present in each cell, but the cell body shows no signs of division.

Fig. 23 Centrifuged thirty minutes, during the second cleavage; fixed six hours later; two of the macromeres are small and protoplasmic; tetrasters are present in the other two.

Fig. 24 Same slide as preceding. All of the macromeres have given rise to normal micromeres of similar size, although two of the macromeres are small and purely protoplasmic while the other two are large and contain much yolk and little cytoplasm.

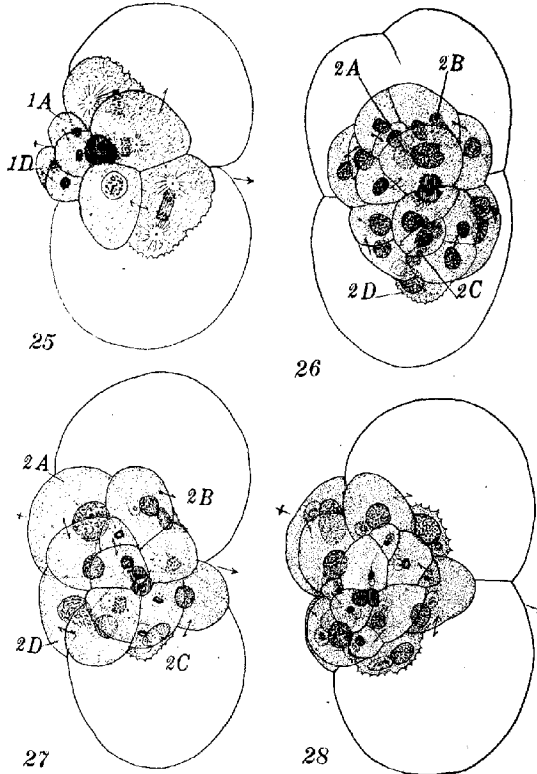


Fig. 25 Same slide as preceding. The minute protoplasmic 'macromeres' (*A* and *D*) have divided equally into the macromeres *1A* and *1D* and the micromeres *1a* and *1d*. The other macromeres (*B* and *C*) have given rise to micromeres somewhat larger than usual.

Fig. 26 Centrifuged for thirty minutes during the second cleavage; fixed twelve hours later; the nuclear divisions of the second cleavage were completed, but the cell divisions were suppressed. Each of these two binucleate macromeres has given rise to two first, and two second quartet cells, just as if four macromeres were present, and each of these micromeres has subdivided in approximately normal manner and is uni-nuclear.

Fig. 27 Centrifuged for thirty minutes in 2-cell stage; fixed six hours later. Micromeres formed from protoplasmic macromeres are of the same size as those formed from large yolk macromeres.

Fig. 28 Same as preceding. The regulation in the formation of micromeres is complete.

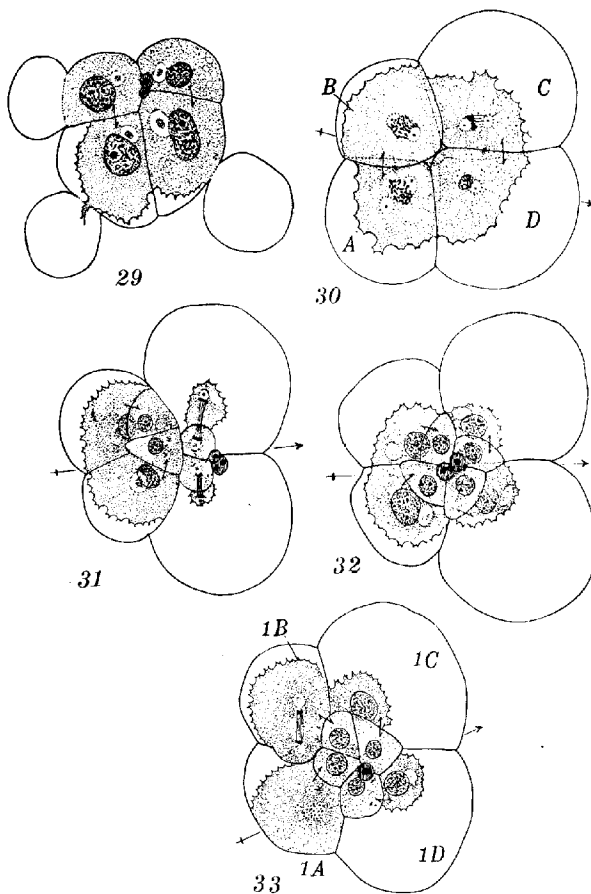


Fig. 29 Centrifuged four hours (2000 revolutions per minute); fixed at once. The yolk was thrown out into lobes, one of which has been detached; the smaller nuclei are in the smaller cells.

Fig. 30 Centrifuged ten minutes in gum arabic during first cleavage; fixed four hours later. Asters and spindles are proportional to the volume of the cytoplasm.

Fig. 31 Centrifuged four hours during the first cleavage; fixed six hours later. Most of the cytoplasm is in the smaller macromeres and these have divided earlier than the larger ones. At least one-half of the cytoplasm in the larger macromeres goes into the micromeres. The first cleavage is not strictly meridional and the spiral form of division is lost. (For explanation of figs. 32 and 33, see p. 98).

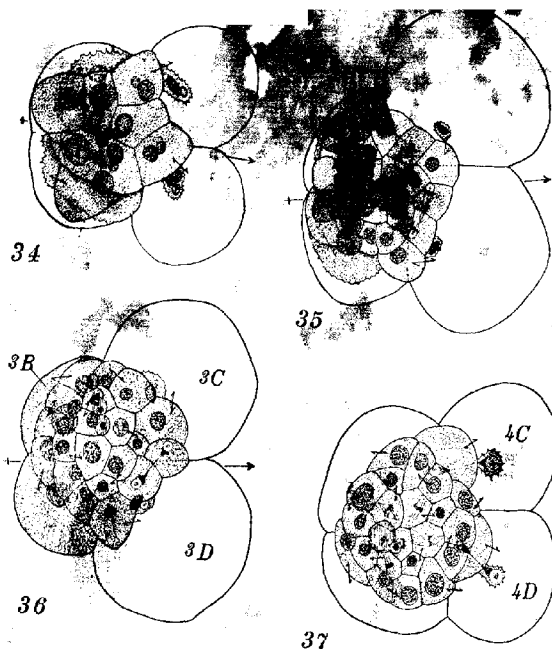


Fig. 32 From the same slide as the preceding. Though the macromeres differ in size and protoplasmic content, the micromeres are all of the same size.

Fig. 33 Centrifuged ten minutes in gum arabic; fixed four hours later. The protoplasmic macromeres are dividing earlier than the others. The size of the micromeres does not depend upon the quantity of cytoplasm in the macromeres from which they came.

Fig. 34 Centrifuged two and one-half hours; fixed twenty-one hours later. The size of the micromeres is almost irrespective of the size of the macromeres; also it is nearly independent of the amount of cytoplasm in the macromeres.

Fig. 35 Centrifuged thirty minutes during the first cleavage; fixed twelve hours later. The micromeres from the protoplasmic macromeres are but little larger than those from the yolk cells.

Fig. 36 From the same slide as the preceding, showing essentially the same conditions.

Fig. 37 From the same slide as the preceding. The cell 4c forms at the same time as 4d, though in normal eggs it is formed much later.

## STUDIES ON THE PHYSIOLOGY OF REPRODUCTION IN THE DOMESTIC FOWL

### V. DATA REGARDING THE PHYSIOLOGY OF THE OVIDUCT<sup>1</sup>

RAYMOND PEARL AND MAYNIE R. CURTIS

FOUR FIGURES

#### INTRODUCTION

The oviduct of a laying hen is divided into five main parts, readily distinguishable by gross observation. Beginning at the cranial end of the organ these parts, in order, are: (a) the infundibulum, or funnel, (b) the albumen secreting portion, (c) the isthmus, (d) the uterus or 'shell gland' and (e) the vagina. Each of these parts is generally supposed (*teste* the existing literature) to play a particular and exclusive rôle in the formation of the protective and nutritive envelopes which surround the yolk in the complete egg as laid. Thus the funnel grasps the yolk at the time of ovulation; the glands of the albumen region secrete the different sorts of albumen (thick and thin) found in the egg; the shell membranes are secreted in the isthmus; and finally the glands of the uterine wall secrete the calcareous shell. This is in brief the classical picture of the physiology of the oviduct.

The gross anatomical appearance and relation of the several parts of the oviduct of the fowl are shown in fig. 1.

<sup>1</sup> Papers from the Biological Laboratory of the Maine Experiment Station, No. 33. The previous papers in this series of "Studies on the Physiology of Reproduction in the Domestic Fowl" are:

i. Regulation in the morphogenetic activity of the oviduct. *Jour. Exp. Zool.*, vol. 6, pp. 339-359, 1909.

ii. Data on the inheritance of fecundity obtained from the records of egg production of the daughters of '200-egg' hens. *Maine Agricultural Experiment Station, Annual Report for 1909*, pp. 49-84.

iii. A case of incomplete hermaphroditism. *Biological Bulletin*, vol. 17, pp. 271-286, 1909.

iv. Data on certain factors influencing the fertility and hatching of eggs. *Maine Agricultural Experiment Station, Annual Report for 1909*, pp. 105-164.

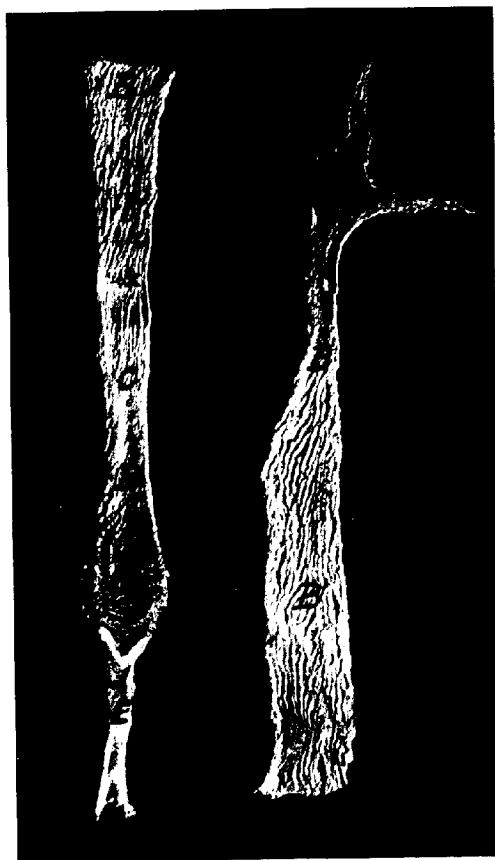


Fig. 1 Photograph of a hen's oviduct which has been removed, slit longitudinally throughout its length and opened out flat in order to show the gross anatomy. In order to get the whole duct on the photographic plate it was necessary to transect it at about the middle. *A*, the infundibulum. Note muscle fibers in wall and absence of any extensive gland development. *B*, albumen secreting portion; note heavy glandular development. The albumen portion ends and the isthmus begins at *x*. The line of demarcation is very distinct in the freshly prepared oviduct. *C*, the isthmus. *D*, the uterus or shell gland. *E*, the vagina; about one-third natural size.

For some years past experiments and observations have been systematically carried on in this laboratory with the object of acquiring a more extended and precise knowledge of the physiology of the hen's oviduct than is to be gained from the literature. It is the purpose of this paper to present a certain part of the results obtained bearing upon the physiology of two of the lower (caudal) morphological divisions of the duct, namely, the isthmus and the uterus. Our results indicate that these portions of the oviduct perform certain functions which have not hitherto been observed or described.

So far as we are able to learn from the existing literature the opinion has been held by all who have worked upon the subject that the particular functional activity of each portion of the oviduct (as above described) is limited to that portion. Thus it is commonly held that when an egg in its passage down the oviduct leaves the albumen portion it has all the albumen it will ever have; when it leaves the isthmus it has all its shell membranes; and when it leaves the uterus all its shell. On this prevailing view there are in the albumen portion only albumen secreting glands; in the isthmus only membrane secreting glands; and in the uterus only shell secreting glands. We were first led to doubt the entire adequacy of this assumption by the observation, frequently made in connection with routine autopsy work, that eggs in the isthmus with completely formed shell membranes, and eggs in the uterus bearing in addition to the complete shell membranes a partially formed shell, weighed considerably less than the normal average for laid Barred Plymouth Rock eggs. This observation led to an inquiry as to whether (a) this apparent lower weight of presumably completed, but not laid eggs, as compared with those which had been laid, was a real phenomenon of general occurrence, and (b) if so, to what it was due. Does the egg increase in weight after the formation of shell membranes and shell merely by the absorption of water, or by the actual addition of new albumen? These are the problems with which the present paper has to do.

We may now turn to the consideration of the observational and experimental data.



## THE DISTRIBUTION OF THE DIFFERENT KINDS OF ALBUMEN IN THE EGG

In the normal egg of the hen there are certainly three and possibly four different albumen layers which can easily be distinguished on the basis of physical consistency. These are: (A) the *chalaziferous layer*. This is a thin layer of very dense albuminous material which lies immediately outside the true yolk membrane. It is continuous at the poles of the yolk with the chalazae, and is undoubtedly found in connection with those structures. It is so thin a layer that it might well be, and often has been, taken for the yolk membrane. (B) The inner layer of *fluid* (thin) *albumen*. This layer is only a few millimeters in thickness and there is some doubt as to its existence as a separate, distinct layer. (C) The *dense albumen*. This is the layer which makes up the bulk of the 'white' of the egg. It is composed of a mass of dense, closely interlaced albumen fibres, with some thin fluid albumen between the meshes of the fibrous network. The dense albumen as a whole will not flow readily, but holds itself together in a flattened mass if poured out upon a plate. (D) The outer layer of *fluid albumen*. This is the principle layer of thin albumen, which makes up the fluid part of the 'white' observed when an egg is broken.

Three of these layers, A, C, and D are readily demonstrable and there can be no question whatever as to their existence. Regarding the existence of B as a separate and distinct layer there is more doubt. Gadow<sup>2</sup> definitely asserts the existence of such a layer in the following words: "Dicht auf der Dotterhaut befindet sich eine dünne Lage des flüssigen Eiweisses." It is possible that what has been taken by previous observers to form this layer B is only a little thin albumen squeezed out of the meshes of the dense layer (C) when the egg is broken.

Let us now consider the distribution of the different sorts of albumen in eggs at different stages in their passage down the oviduct. The following extracts from autopsy protocols are to the point here.

<sup>2</sup> Gadow, H., Vogel (Anatomischer Theil); in Bronn's Klassen und Ordnungen des Thier-Reichs. Leipzig, 1891, p. 869.

*Autopsy No. 370. Hen No. 952. March 19, 1910*

Egg found in the albumen portion of the oviduct 11 cm. in front of the cranial end of the isthmus. This egg consisted of a yolk surrounded by thick albumen (layer C) but with no trace whatever of the albumen layer D. Not yet having entered the isthmus the egg lacked a shell membrane.

*Autopsy No. 332. Hen No. 420. December 22, 1909*

This case was similar to that just cited. Here an egg was in the albumen portion of the duct with its caudal end 6 cm. in front of the cranial end of the isthmus. The egg consisted of yolk surrounded by dense albumen (layer C). There was no trace of the thin albumen (layer D) to be observed. The egg had no shell membrane.

*Autopsy No. 366. Hen No. 276. March 18, 1910*

Egg in albumen portion of oviduct with its caudal end 4 cm. in front of the cranial end of the isthmus. This egg had no shell membrane. The yolk was surrounded by thick albumen (layer C). The egg bore no trace of the thin albumen (layer D), even though it was only this short distance (4 cm.) from the point where the 'albumen secreting' portion of the duct was finally to be left.

*Autopsy No. 301. Hen No. E39. July 14, 1909*

When this bird was killed an egg was found at the lower end of the albumen portion of the oviduct just about to enter the isthmus. Not yet having entered the isthmus the egg had no shell membrane upon it. It consisted merely of a yolk surrounded by albumen. The outermost layer of this albumen was dense and corresponded to layer C described above. There was no trace of thin albumen (layer D) on this egg although it was just on the point of leaving the so-called albumen region of the oviduct.

*Autopsy No. 369. Hen No. 1154. March 19, 1910.*

**Egg** in the lower end of the albumen portion of the oviduct just at the point of entering the isthmus. This egg had no membrane. The yolk was surrounded by albumen layers A, B, and C. No trace of the outer thin albumen (layer D) was to be found.

All these cases agree in showing that *the egg does not receive the outer layer of thin fluid albumen (layer D) during its sojourn in the so-called albumen secreting portion of the oviduct.* While but five specific autopsy records are cited here it is only fair to say that this result is confirmed by all our experience with eggs in the albumen secreting portion of the oviduct. This experience covers many more than the five cases given here. These cases are chosen as particularly significant, however, because in them we have definite quantitative records of the exact location of the egg in the albumen portion. The successive autopsy records show that beginning with an egg 11 cm. away in front of the isthmus and going downwards in the duct until the actual boundary of the isthmus is reached, there is no qualitative change in the albumen secretion. Whatever albumen is added to the egg immediately prior to the formation of the shell membrane, is of the dense fibrous variety (layer C), so far as direct observation indicates. The fact that there is no thin albumen on the egg when it enters the isthmus is shown in fig. 2. In this figure A shows an egg of hen No. 8018 which was removed from the upper part of the isthmus. The thin membrane which had been formed was removed; the egg placed in a Petri dish and photographed. For comparison a normal laid egg from hen 8018 was broken into a Petri dish in the same way. Its photograph is shown in fig. 2, B. It is at once apparent that there is a great difference between the two eggs in respect to amount and consistency of albumen. In the egg which had just left the albumen portion of the oviduct (egg A) the albumen is of firm consistency and retains its shape, forming a compact mass about the yolk. In the laid egg (egg B) the albumen is much thinner, and does not hold its shape, but flows out over the bottom of the dish.

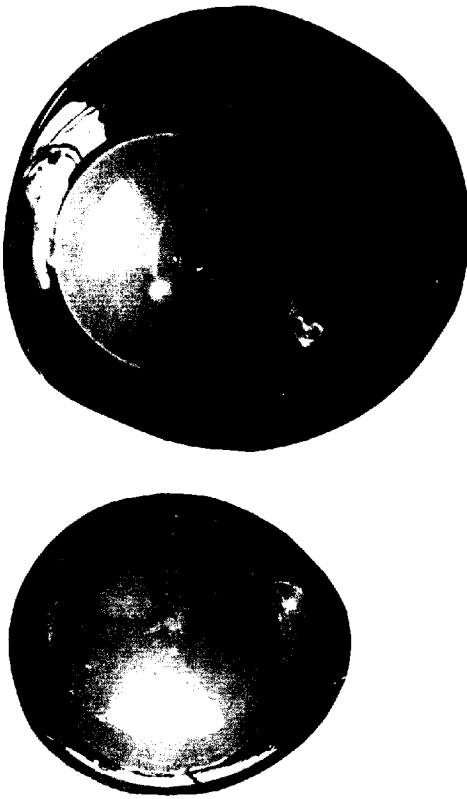


Fig. 2. Showing *A* an egg taken from the isthmus of the oviduct of hen No. 8018, with its shell membrane removed, and *B* a normal laid egg of the same bird, photographed under identical conditions; natural size. For further explanation see text.

Crucial evidence is here afforded by those cases occasionally to be observed, where the egg is just entering the isthmus, and has one end in the albumen portion of the duct and the other end in the isthmus. It was first pointed out by Coste<sup>3</sup> that the formation of the shell membrane at the upper end of the isthmus is a discrete process. That is, as the end of the egg advances from the albumen portion into the isthmus, membrane is deposited upon it. The membrane is complete over the whole egg only after the egg has entirely passed into the isthmus. This account of membrane deposition we have confirmed by direct observation in this laboratory. Now in cases where one-half of the egg lies within the isthmus and bears a membrane while the other half is in the albumen portion and has no membrane it can plainly be seen that *the shell membrane is deposited directly on the outer surface of the thick albumen (layer C) and that no trace of the thin albumen (layer D) is present at the time the membrane is formed.*

It might be contended that the thin albumen which is to form layer D is really present at the time the membrane is deposited, but that instead of forming a separate outer layer it is held by adhesion or otherwise within the meshes of the fibrous network of the dense albumen of layer C. On this view it might be supposed that this more fluid albumen passes out of the network to form a definite and separate layer at some time after the membrane is laid down. This contention, however, cannot be correct, because, as will be demonstrated in the next section of the paper, the egg does not have its full complement of albumen by weight at the time when the shell membrane is formed. The fluid albumen of layer D should weigh just as much, whether in the interstices of a fibrous meshwork, or forming a separate layer. Yet the facts show that after a thin albumen (layer D) has been *visibly* formed the egg contains by weight about 50 per cent more albumen than it did before this layer was *visibly* formed.

<sup>3</sup>Coste, M., Histoire du développement des corps organisés, tome 1, p. 295, 1847.

THE PROPORTIONATE WEIGHT OF YOLK AND ALBUMEN IN EGGS  
IN DIFFERENT STAGES OF FORMATION

Having learned by direct observation, as set forth in the preceding section that the egg as it enters the isthmus does not visibly bear the outer layer of thin albumen, the next step in the analysis is to determine whether the amount of albumen (by weight) in the egg definitely increases during its sojourn in the isthmus and uterus, and if so to what extent. In order to do this it is necessary to take eggs at successive intervals after they have entered the isthmus, separate and weigh yolk and albumen each by itself, and then compare the weights so obtained with the weights of yolk and albumen in normal, completely formed and laid eggs produced by the same individual birds. Experiments of this kind we have carried out with the results described in this section of the paper.

It should be said that the technique followed in the separating and weighing of the eggs to furnish these data is that described by one of the authors in another place.<sup>4</sup>

Table 1 gives data regarding the weight of yolk and albumen in eggs which have completed their passage through the albumen secreting portion of the oviduct, and have advanced varying distances into the isthmus and shell gland. The data here given are extracted from the more detailed table exhibited in the Appendix of this paper.

The plan of table 1 is to compare the weights of the parts of a series of eggs taken from different levels of the oviduct with the weights of the same parts in normal laid eggs of the same birds. Owing to the considerable individual variability in the weights of eggs it is only by such comparisons as this that reliable results may be reached. To determine the means for the normal laid egg varying numbers of eggs were used in different cases. In one instance (item 3) only one laid egg was available for comparison. In all other cases the mean of two or more complete normal, laid eggs are used. It will be noted that in somecases

<sup>4</sup> Curtis, M. R., Annual Report Maine Agricultural Experiment Station, 1911, pp. 93-112.

(items 3, 4, 6, 9, and 15) the weights are given only to one decimal place (tenths of a gram). These were eggs studied in the early stage of the investigation, and mostly are cases in which the data were taken in connection with other studies in progress in the laboratory, for which finer weighing was not essential. These cases are to be regarded as giving a much rougher sort of data than the others tabled, where the weighings are accurate to hundredths of a gram. In no instance, however, does one of these 'rough' cases stand alone. That is, there are one or more eggs for which finer weighing are tabled from each of the levels of the oviduct wherefrom a roughly weighed egg was taken. These 'rough' cases then serve merely to confirm evidence obtained from more precise weighing. All differences in the table are given the + sign when the oviduct egg or its part is greater than the laid egg or its part. The differences are taken - when oviduct egg or its part is smaller. The last column of the table gives the percentage which the weight of albumen in the oviduct egg at the specified level is of the mean total weight of albumen in the normal laid egg of the same bird. This last column then shows directly what proportion of the total albumen which the egg is to have has been laid down at each specified level of the oviduct.

From table 1 the following points are to be noted:

1. When the egg leaves the albumen portion of the oviduct it weighs roughly only about half as much as it does when it is laid. Nearly all of this difference is in the albumen. Thus these weighings fully confirm the conclusion reached from direct examination of the eggs, as described in the preceding section. The evidence thus far presented shows that the egg gets all of its thin albumen (layer D), which constitutes nearly 60 per cent by weight of the total albumen, only after it has left the supposedly only albumen secreting portion of the oviduct, and has acquired a shell membrane, and the shell is in process of formation. The fact that the egg increases considerably in size after it enters the isthmus is obvious from simple visual comparison of egg from this region of the oviduct with normal laid eggs of the same bird even though no weights whatever are taken.

TABLE 1

Data showing the increase in absolute and relative weight of albumen after the egg has passed through the so-called albumen secreting part of the oviduct

LOCATION OF EGG IN OVIDUCT	TOTAL WEIGHT	WEIGHT OF SHELL AND MEMBRANES	WEIGHT OF YOLK	WEIGHT OF ALBUMEN	100 X ALBUMEN YOLK	PER CENT ALBUMEN
	grams	grams	grams	grams		
1. At caudal end of albumen portion. No membrane (Hen 8009).....	27.20	0	15.38	11.82	76.9	34.2
Mean of the four previously laid eggs of same hen.....	57.57	6.23	16.79	34.55	205.8	
Difference.....	-30.37	-6.23	-1.41	-22.73	-128.9	
2. At caudal end of albumen portion. No membrane (Hen 8005).....	29.53	0	15.87	13.66	86.1	43.6
Mean of the nine previously laid eggs of same hen.....	52.81	5.79	15.67	31.34	200.0	
Difference.....	-23.28	-5.79	+0.20	-17.68	-113.9	
3. Just entering isthmus, little cap of membrane on caudal tip. (Hen 1293).....	29.5	0	16.0	13.5	84.4	43.5
Mean of two previously laid eggs of same hen.....	56.25	9.0	16.25	31.0	190.8	
Difference.....	-26.75	9.00	-0.25	-17.5	-106.4	
4. Entering isthmus. Covered with membrane except for a little of cranial tip. (Hen 266F).....	31.0		14.5	16.5	113.8	53.2
Normal egg laid by hen same day.....	51.0	6.0	14.0	31.0	221.4	
Difference.....	-20.0		+0.5	-14.5	-107.6	
5. Entering isthmus. Covered with membrane except for a little of cranial tip. (Hen 8027).....	32.44	0.24	15.69	16.51	105.2	51.5
Mean of the four previously laid eggs of same hen.....	54.69	5.76	16.87	32.07	190.2	
Difference.....	-22.25	-5.52	-1.18	-15.56	-85.0	



TABLE 1—Continued

LOCATION OF EGG IN OVIDUCT	TOTAL WEIGHT	WEIGHT OF SHELL AND MEMBRANES	WEIGHT OF YOLK	WEIGHT OF ALBUMEN	100 × ALBUMEN YOLK	PER CENT ALBUMEN
	grams	grams	grams	grams		
6. In upper part of isthmus. Membrane complete, but thin. (Hen 2554).....	32.0		16.5	15.5	93.9	48.1
Mean of two previously laid eggs of same hen. ....	60.5	8.75	19.5 <sup>6</sup>	32.25	165.4	
Difference.....	-28.5		-3.0	-16.75	-71.5	
7. In upper part of isthmus. Membrane thin. (Hen 8008).....	28.33	0.28	12.57	15.48	123.2	49.5
Mean of the five previously laid eggs of same hen. ....	50.20	5.54	13.39	31.27	233.5	
Difference.....	-21.87	-5.26	-0.82	-15.79	-110.3	
8. Three cm. below beginning of isthmus. Membrane thin. (Hen 1367).....	32.08	0.39	16.12	15.57	96.6	56.9
Mean of nine previously laid eggs of same hen. ....	48.45	5.40	15.70	27.34	174.1	
Difference.....	-16.37	-5.01	+0.42	-11.77	-77.5	
9. Two cm. above caudal end of isthmus. (Hen 416).....	31.0	2.0	16.0	13.0	81.3	50.5
Mean of two previously laid eggs of same hen. ....	47.5	7.0	14.75	25.75	174.6	
Difference.....	-16.5	-5.0	+1.25	-12.75	-93.3	
10. In lower part of isthmus. (Hen 8010).....	30.00	0.53	14.04	15.43	109.9	51.3
Mean of eleven previously laid eggs of same hen. ....	51.54	5.77	15.72	30.06	191.2	
Difference.....	-21.54	-5.24	-1.68	-14.63	-81.3	
11. In lower part of isthmus. (Hen 8018).....	37.27	0.58	16.37	20.32	124.1	56.4
Mean of four previously laid eggs of same hen. ....	60.10	6.63	17.42	36.05	206.9	
Difference.....	-22.83	-6.05	-1.05	-15.73	-82.8	

<sup>6</sup> This is too high a value, probably arising from errors in separation of yolk and white. These particular data were taken before the refinement of method used in later studies had been worked out.

TABLE 1—Continued

LOCATION OF EGG IN OVIDUCT	TOTAL WEIGHT	WEIGHT OF SHELL AND MEMBRANES	WEIGHT OF YOLK	WEIGHT OF ALBUMEN	$100 \times \frac{\text{ALBUMEN}}{\text{YOLK}}$	PER CENT ALBUMEN
	grams	grams	grams	grams		
12. In uterus, but no shell found. Egg surrounded by fluid in uterus. (Hen 8038).....	43.93	0.76	17.30	25.87	149.5	71.1
Mean of four previously laid eggs of same hen....	60.92	6.76	17.77	36.39	204.8	
Difference.....	-16.99	-6.00	-0.47	-10.52	-55.3	
13. In uterus, but no visible shell formed. (Hen 8030).....	45.15	0.96	17.25	26.91	156.2	75.5
Mean of the six previously laid eggs of same hen....	59.22	6.22	17.34	35.66	205.7	
Difference.....	-14.07	-5.26	-0.09	-8.72	-49.5	
14. In uterus but no visible shell formed. Some fluid in uterus. (Hen 8033).....	46.67	0.88	19.13	26.66	139.4	80.7
Mean of two previously laid eggs of same hen....	58.99	6.91	19.04	33.04	173.5	
Difference.....	-12.32	-6.03	+0.09	-6.38	-34.1	
15. In uterus, small amount of shell formed. (Hen 1061).....	45.00	4.50	15.50	25.00	161.3	87.0
Mean of two previously laid eggs of same hen....	52.00	7.50	15.75	28.75	182.5	
Difference.....	-7.00	-3.00	-0.25	-3.75	-21.2	
16. In uterus, some shell formed. (Hen 8021)....	43.66	1.35	15.18	27.13	178.7	95.1
Mean of three previously laid normal eggs of same hen.....	49.26	5.18	15.56	28.52	183.3	
Difference.....	-5.60	-3.83	-0.38	-1.39	-4.6	

Thus in fig. 3 are shown (1) a membrane covered egg taken from the isthmus of the oviduct of hen No. 8018 shortly before it would have entered the uterus, and (2) two normal laid eggs of the same bird. The larger size of the latter is obvious. The isthmus egg is the one at the extreme left in the picture.

2. It is apparent from examination of the differences in the columns giving albumen weights and albumen-yolk ratios that in general the farther down the oviduct the egg proceeds the more albumen it gets. Very nearly one-half the total weight of albumen of the completed egg is added in the uterus, an organ hitherto supposed to be entirely devoted to shell formation. Clearly very much more albumen is added to the egg in the uterus than in the isthmus. This, of course, does not necessarily mean any more rapid rate of secretion in the uterus, because of the time element involved. The egg stays much longer in the uterus than in the isthmus.

3. This brings us to a consideration of the question of the rate of secretion of albumen in different positions of the oviduct. We have attempted to approach this problem by the graphical method. The results obtained are not to be regarded as highly accurate in respect to minute details. It is an exceedingly difficult matter to get very precise data in the individual instances regarding time relations in the physiology of the oviduct. We must therefore depend upon average results. The attempt has been made in fig. 4 to show graphically the net average results from the data collected in this laboratory regarding the time taken in the passage of the egg through the several portions of the oviduct and the rate of secretion of albumen in the same portions. As a measure of the albumen is taken the percentage of the total albumen of the laid egg which has been acquired at each specified level of the duct. The time is plotted as abscissa, and the percentage of albumen as ordinate.

It is not possible to recount here in detail all the evidence on which the points in this diagram are based. It would involve the presentation of considerable material which has no direct bearing on the subject of the present paper. We shall, therefore, be obliged to state only briefly, and in some degree categorically,

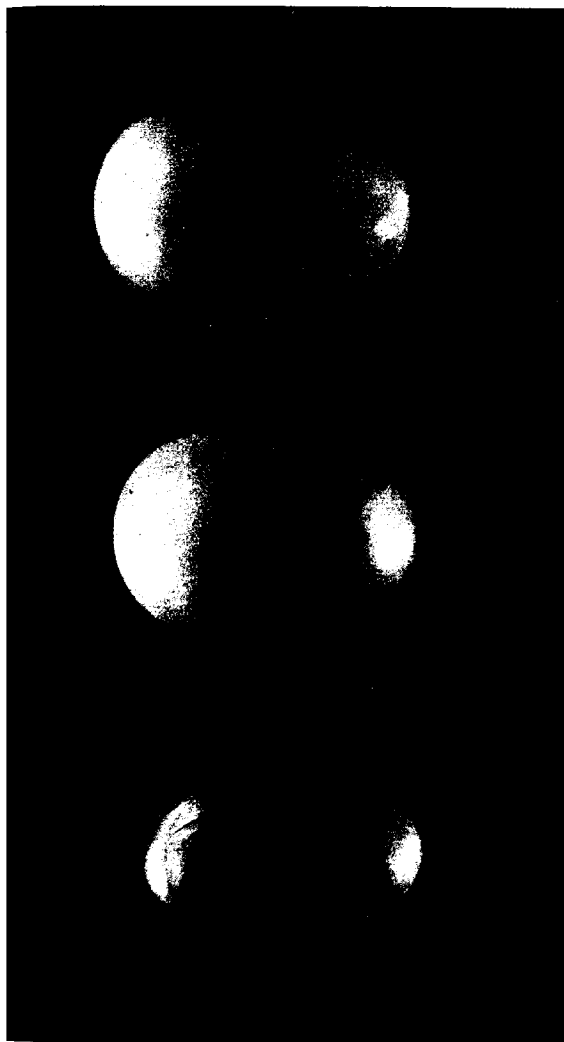


Fig. 3. Showing a membrane covered egg from the isthmus (then SOLS) at the left and two normal laid eggs of the same bird for comparison; natural size; (see text).

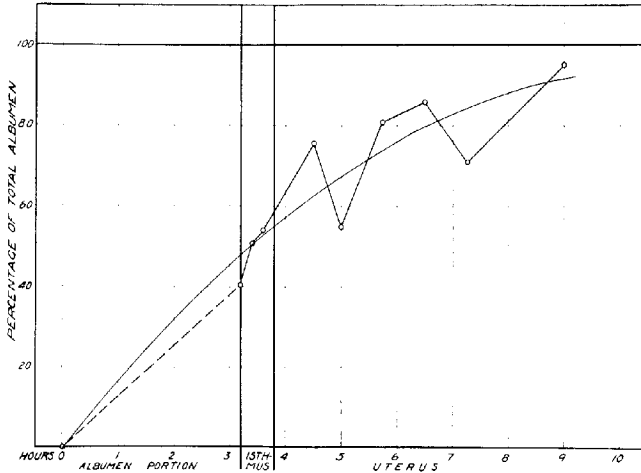


Fig. 4. Diagram showing what percentage of the total amount of albumen present in the normal laid egg of the domestic fowl is present at successive levels in the oviduct. The smooth curve is the parabola for which the equation is given in the text.

the manner in which the plotted points were determined. In the first place it has been found in our work here that when a hen is laying regularly one egg per day ovulation occurs at approximately the same time as laying. That is the oöcyte which will be laid as a completed egg tomorrow enters the infundibulum at the time when today's passes through the vagina and is laid. This then is taken as a fundamental datum in the calculation of the rate of passage of the egg down the oviduct.

The mean of all available observations made in this laboratory gives 3.2 hours as the time required for the passage of the egg through the 'albumen portion' of the oviduct. This includes the total time from the entrance of the egg into the infundibulum to its entrance into the isthmus. This agrees very well with the statements of earlier workers<sup>6</sup> who generally give the time spent in the albumen portion of the duct as 'about' three hours.

<sup>6</sup> Cf. Lillie, F. R. *The Development of the Chick*. New York, 1908, pp. 23-25.

In regard to the time taken by the egg in passing through the isthmus, our observations are far from agreeing with the statements on this point in the literature. Taking the mean of all available data 0.6 of an hour is found to cover the time during which the egg is in the isthmus. All our observations agree well amongst themselves, and we are convinced that this figure is substantially correct for the breed of fowls here used (Barred Plymouth Rocks). This is a much shorter time than earlier workers have estimated. Thus Gadow<sup>7</sup> says: "Im Isthmus soll das Ei ungefähr 3 Stunden lang verweilen." Lillie<sup>8</sup> gives the same estimate on the authority of Kölliker. Patterson<sup>9</sup> who has published most recently on this matter, while reducing somewhat the time for passage through the isthmus, still gives a value considerably higher than that found in the work of this laboratory. His statement is as follows (loc. cit., p. 105): "The writer finds that in a hen kept under normal conditions, the egg traverses the entire length of the oviduct in about twenty-two hours. The time occupied in the different portions of the oviduct is as follows: Glandular portion, three hours; isthmus, two to three hours; uterus and laying sixteen to seventeen hours." With all parts of this statement except that relating to the isthmus our results are in entire agreement. At an early stage of the studies in this laboratory on the physiology of the oviduct we were of the same opinion as Patterson as to the time taken in passing through the isthmus. More extended observations, covering a fairly wide range of conditions has convinced us that, as already stated, the egg normally takes less than one hour in passing through the isthmus. It is, of course, possible that there are breed differences in respect to the time the egg stays in the isthmus, and that Barred Plymouth Rocks are strikingly exceptional in this regard but this hardly seems probable. It is more likely that the estimate of earlier workers has been somewhat too large.

<sup>7</sup> Gadow, H., loc. cit., p. 872.

<sup>8</sup> Loc. cit.

<sup>9</sup> Patterson, J. T., *Journal of Morphology*, vol. 21, pp. 101-134, 1910.

As stated in the preceding paragraphs the points on the time or abscissal axis represent the mean or average results of fairly extensive experimental data, some of which are not included in the present paper. Now we may consider the determination of the points plotted as ordinates. At the outstart it should be said that no observations have yet been made on the rate of secretion of albumen at different levels of the 'albumen portion' itself. Therefore from the zero point at 'ovulation' when the naked yolk enters the infundibulum to the beginning of the isthmus we have connected the points with a dotted line to indicate that in this region no direct observations are available. The first plotted point (40.4) at the beginning (cranial end) of the isthmus is the mean albumen percentage of three eggs which were taken from this point in the duct. The next point plotted is the mean albumen percentage (50.6) of four isthmus eggs which were taken from the upper part of the isthmus. The next point (53.8) is the mean albumen percentage of four isthmus eggs taken from the lower part of the isthmus. The last six points are based on the observations of single eggs which have been in the uterus the indicated length of time.

The smooth curve which graduates these observations is the parabola

$$y = 17.5915x - 0.8171x^2 - 0.4164,$$

in which  $y$  denotes percentage of albumen and  $x$  time in hours during which the egg has been in the oviduct. The origin of  $x$  is taken at  $O$  (ovulation). The parabola was fitted to the observations by the method of least squares.

The diagram shows clearly that there is scarcely any diminution in the rate of secretion of albumen until nearly the total amount has been acquired by the egg. There is not the slightest evidence of any break in the rate of secretion of albumen after the egg leaves the so-called 'albumen portion' of the duct. From the time the yolk enters the upper end of the 'albumen portion' there is a gradual diminution of the rate of secretion of albumen, giving rise to the parabolic curve. But plainly there is no sudden change. The egg gets more than half of its total albumen after

it leaves the 'albumen portion' of the duct and it takes this at nearly the same rate as it did the earlier part.

It is of interest to note the similarity of this curve showing the rate of increase of albumen in the formation of the individual egg to the curve previously published by one of the writers<sup>10</sup> for the increase in weight of egg (which is quite closely correlated with amount of albumen) with increasing amount of yolk, as measured by number of yolks.

4. It will be noted that the differences in the column headed 'weight of yolk' are in the majority of cases negative. In other words, in these instances the yolk of the laid egg is heavier than the yolk of the oviduct egg. Now, of course, yolk as such is not added during the passage of the egg down the oviduct. This being so one would expect that in the long run the yolk of any given laid egg would be as often in defect as in excess of the weight of the yolk of any given oviduct egg. There is some indication that this is not strictly true, but that the 'laid' yolk tends to be heavier. Such a phenomenon would be in accord with the fact definitely demonstrated by Greenlee,<sup>11</sup> in his study of cold storage eggs, that in the normal, unboiled egg there is a continuous transfer of water from albumen to yolk by osmosis. Certain of Miss Curtis'<sup>12</sup> earlier results had suggested that this was possibly the case.

#### THE ABSOLUTE AND RELATIVE AMOUNT OF NITROGEN IN THE ALBUMEN OF EGGS IN DIFFERENT STAGES OF FORMATION

While the two lines of evidence presented in the preceding sections of the paper amply demonstrate that the thin albumen is added to the egg after it leaves the albumen portion of the duct, it seemed advisable, because of the novelty of the results to collect still further evidence of another kind. This evidence, which will be set forth in the present section of the paper, has to do with

<sup>10</sup> Pearl, R., *Zoologischer Anzeiger*, Bd. 35, pp. 417-423, 1910.

<sup>11</sup> Greenlee, A. D., U. S. Department Agriculture Bureau of Chemistry, Circular 83, pp. 1-7, 1911.

<sup>12</sup> Curtis, M. R., loc. cit.



the nitrogen content of the albumen in eggs taken from different levels of the oviduct. Not only do these chemical data confirm the results obtained from the other lines of evidence, thus demonstrating still more thoroughly and conclusively the main point under discussion, but they also throw light on certain matters which could not be elucidated by other than chemical methods of attacking the problem.

The analytical work on which the data of this section are based was performed in the Department of Chemistry of the Maine Experiment Station, by Mr. H. H. Hanson, Associate Chemist. It is a pleasure to express our obligation to him for coming to the aid of the present investigation in this way.

It should be stated that the nitrogen determinations were made by the modified Kjeldahl method, as used by the Association of Official Agricultural Chemists.

The chemical data are exhibited in condensed form in table 2. The complete figures for the same eggs are given in table A of the Appendix.

It is evident that the data set forth in table 2 confirm the results previously obtained. Thus, to consider first moisture content of the albumen, it is seen that the albumen of the normal laid egg contains between 87 and 88 per cent of moisture (mean 87.40). This value agrees very well with those obtained by Willard and Shaw<sup>14</sup> and Pennington.<sup>15</sup> But when the egg enters the uterus its albumen has a water content of only about 80 per cent. Or, the albumen of the egg at this level of the oviduct has by actual weight some 15 grams less water than the laid egg. The longer the egg stays in the uterus the 'thinner' the albumen becomes (cf. hen 387 in the above table), i.e., the higher its water content. This, of course, is the same thing which has been shown above, namely, that most of the thin albumen is added in this region.

The percentage content of the albumen in nitrogen brings out again the same point. From a nitrogen content of about 4 per

<sup>14</sup> Willard, J. T. and Shaw, R. H., Kansas Agricultural Experiment Station, Bulletin 159, pp. 143-177, 1909.

<sup>15</sup> Pennington, M. E., Journal of Biological Chemistry, vol. 7, pp. 109-132, 1910.

cent at the upper end of the isthmus, the relative amount of this element in the albumen diminishes steadily till the egg is laid.

The point of greatest interest and importance in connection with these chemical data, hinges upon the *absolute* amount of nitrogen in the albumen. Since it is solely the thin albumen layer which is added after the egg leaves the albumen portion of the oviduct the possibility is at once suggested that what happens in the lower portions of the duct is not a true secretion of another albumen layer but merely a taking up of water from the blood by osmosis, and a dilution or partial solution of the dense albumen already present. Such a view assumes in other words that all that is added to the albumen after the egg enters the isthmus is water.

Clearly the only way to test finally the validity of this idea is to carry out such chemical determinations as are tabled above. The last column of table 2 shows the available evidence, which appears reasonably clear in its significance, though because of the minute absolute amount of nitrogen in the white of an egg the case is not a simple one. What the figures from the analyses of thirteen oviduct eggs show is that with four exceptions, the oviduct egg has absolutely less nitrogen in its albumen than the normal laid egg of the same hen. This, of course, is what would be expected if there is an actual secretion of albumen by the glands of the oviduct, and this secretion is added to the egg. It means that these oviduct eggs have been removed before they received their full amount of albumen. If it were the case, on the contrary, that only water was added to the egg after it left the albumen portion of the duct, it would be expected that the amount of *nitrogen* would be the same in an oviduct egg from the isthmus or uterus as in the normal laid egg. *The chemical data clearly indicate that there is a definite addition of albumen to the egg in the isthmus and shell gland, and that the thin albumen layer does not represent solely a dilution of the dense layer.*

The four cases in which the analyses furnish an exception to this rule are undoubtedly to be explained as the result of fluctuation in the absolute size of the egg. It will be noted that each of these four eggs have been in the uterus some time and would therefore

TABLE 2

*Data showing the relative and absolute amounts of nitrogen in the albumen of eggs taken from different levels of the oviduct, and in normal laid eggs*

LOCATION OF EGG IN OVIDUCT	WEIGHT OF ALBUMEN	PER CENT OF WATER IN ALBUMEN	WEIGHT OF WATER IN ALBUMEN	PER CENT OF NITROGEN IN ALBUMEN	WEIGHT OF NITROGEN IN ALBUMEN <sup>13</sup>
At caudal end of albumen portion. No membrane. (Hen 8005).....	13.66			4.13	0.5635
Mean of normal laid eggs of same hen.....	31.34			1.98	0.6188
Difference.....	-17.68			+2.15	-0.0553
Entering isthmus.....					
Covered with membrane except for a little of cranial tip. (Hen 8027).....	16.51			3.78	0.6233
Mean of normal laid eggs of same* hen.....	32.07			2.00	0.6381
Difference.....	-15.56			+1.78	-0.0148
Entering isthmus. Covered with membrane except for a little of cranial tip. (Hen 168).....	21.4015	79.45	17.0036	3.04	0.6515
Normal laid egg of same hen.....	38.4895	87.44	33.6536	1.78	0.6860
Difference.....	-17.0880	-7.99	-16.6500	+1.26	-0.0345
In upper part of isthmus. Membrane thin. (Hen 8008).....	15.48			3.47	0.5372
Mean of normal laid eggs of same hen.....	31.27			2.06	0.6407
Difference.....	-15.79			+1.41	-0.1035
In lower part of isthmus. (Hen 8010).....	15.43			3.30	0.5092
Mean of normal laid eggs of same hen.....	30.06			1.90	0.5894
Difference.....	-14.63			+1.40	-0.0802
In lower part of isthmus. (Hen 8018).....	20.32			3.34	0.6756
Mean of normal laid eggs of same hen.....	36.05			1.89	0.6960
Difference.....	-15.73			+1.45	-0.0204

<sup>13</sup> In calculating the absolute amount of nitrogen in the normal laid eggs the mean albumen weight for the whole number of such eggs available for each hen has been used.

TABLE 2—Continued

LOCATION OF EGG IN OVIDUCT	WEIGHT OF ALBUMEN	PER CENT OF WATER IN ALBUMEN	WEIGHT OF WATER IN ALBUMEN	PER CENT OF NITROGEN IN ALBUMEN	WEIGHT OF NITROGEN IN ALBUMEN
In uterus, but no visible shell formed. (Hen 117).....	18.841	79.58	14.9943	3.03	0.5711
Normal laid egg of same hen.....	34.419	87.46	30.1016	1.79	0.6154
Difference.....	-15.578	-7.88	-15.1073	+1.24	-0.0443
In uterus, but no shell formed. Egg surrounded by fluid in uterus. (Hen 8038).....	25.87			2.78	0.7179
Mean of normal laid eggs of same hen.....	36.39			1.78	0.6492
Difference.....	-10.52			+1.00	+0.0687
In uterus, but no visible shell formed. (Hen 8030).....	26.94			2.50	0.6727
Mean of normal laid eggs of same hen.....	35.66			1.79	0.6485
Difference.....	-8.72			+0.71	+0.0242
In uterus, but no visible shell formed. Some fluid in uterus. (Hen 8033).....	26.66			2.30	0.6118
Mean of normal laid eggs of same hen.....	33.04			1.68	0.5534
Difference.....	-6.38			+0.62	+0.0684
In uterus, small amount of shell. (Hen 200).....	21.371	82.89	17.7154	2.51	0.5371
Normal laid egg of same hen.....	27.608	86.80	23.9627	1.88	0.5197
Difference.....	-6.237	-3.91	-6.2473	+0.63	+0.0174
In uterus. Small amount of shell. (Hen 387).....	29.9665	86.62	25.9559	1.92	0.5741
Normal laid egg of same hen.....	34.9305	87.89	30.7005	1.72	0.5997
Difference.....	-4.9640	-1.27	-4.7446	+0.20	-0.0256
In uterus. Some shell formed. (Hen 8021).....	27.13			2.07	0.5616
Mean of normal laid eggs of same hen.....	28.52			1.96	0.5653
Difference.....	-1.39			+0.11	-0.0037

have received nearly their full amount of albumen. It can readily be seen that if the oviduct egg happens to be an exceptionally large one, relative to the other eggs of the same bird, it may have a slightly greater absolute amount of nitrogen though not yet laid, than another relatively or absolutely smaller laid egg taken for a control. It seems quite clear, in the light of data collected in this laboratory on the fluctuation in size and proportions of the parts of eggs<sup>16</sup> that this is the correct explanation of these apparent exceptions in the chemical analyses. It will be noted that in these four cases it is only the *absolute* amounts of nitrogen (and not the *percentages*) which furnish exceptions to the rule.

*Supplementary evidence*

There is available evidence of still other sorts to indicate that there is a real addition of albumen to the egg after it leaves the so-called 'albumen' portion of the oviduct. In the first place a histological study of the oviduct which has been made in this laboratory by Dr. Frank M. Surface<sup>17</sup> shows that histologically the same kind of glands which are found in the so-called albumen portion of the duct, are also found in the isthmus and uterus. The differences between the glands of the different regions are quantitative not qualitative.

In removing eggs from the uterus it is frequently found that the egg is surrounded by a thin fluid which has evidently been secreted and is in process of being taken into the egg by osmosis. A case of this sort is described in the following autopsy record.

*Autopsy No. 523. Hen 8038. Killed March 28, 1911 for data*

This hen laid at 9 A.M. and was killed at 4.15 P.M., or 7½ hours after laying. There was an egg in the uterus. The uterus was much larger than the egg. When a cut was made in this organ a small amount of clear fluid flowed out. The cut was clamped off

<sup>16</sup> Cf. No. 1 of these 'Studies,' loc. cit. supra.

<sup>17</sup> Reported at the Ithaca meeting of the American Society of Zoologists, Eastern Branch, December, 1910, but not yet published.

and about 2 cc. of the fluid drained into a bottle. This fluid was analysed, the following being the chemist's report:

Amount taken: 1.9860 grams being all of sample.  
Nitrogen found: 0.22 per cent.

From this record it is clear that the fluid taken up by the egg in the uterus, is far from being water. It carries more than a fifth of 1 per cent of nitrogen. In other words it is a dilute albumen.

That the egg does not take water from the blood by osmosis, and in this way dilute the dense albumen to form the thin is further evidenced by the fact, shown by Atkins<sup>18</sup> that in the domestic fowl the osmotic pressure of the blood is very considerably higher (nearly two atmospheres) than the osmotic pressure of the egg. In any osmotic exchange under these conditions water would tend to pass from the egg to the blood and not in the other direction.

#### SUMMARY OF RESULTS

Putting all the evidence together, the following account of the processes by which the hen's egg acquires its protective and nutritive coverings summarizes the results of the present study. Certain of these results are novel and others confirm the experience of earlier workers.

1. After entering the infundibulum the yolk remains in the so-called albumen portion of the oviduct about three hours and in this time acquires only about 40 to 50 per cent by weight of its total albumen and not all of it as has hitherto been supposed.

2. During its sojourn in the albumen portion of the duct the eggs acquires its chalazae and chalaziferous layer, the dense albumen layer, and (if such a layer exists as a distinct entity, about which there is some doubt) the inner fluid layer of albumen.

3. Upon entering the isthmus, in passing through which portion of the duct something under an hour's time is occupied instead of three hours as has been previously maintained, the egg receives its shell membranes by a process of discrete deposition.

<sup>18</sup> Atkins, W. R. G., Scientific Proceedings of the Royal Dublin Society, vol. 12 (N. S.) pp. 123-130, 1909. Cf. also Biochemical Journal, vol. 4, pp. 480-484, 1909.

4. At the same time, and during the sojourn of the egg in the uterus, it receives its outer layer of fluid or thin albumen which is by weight 50 to 60 per cent of the total albumen.

5. This thin albumen is taken in by osmosis through the shell membranes already formed. When it enters the egg in this way it is much more fluid than the thin albumen of the laid egg. The fluid albumen added in this way dissolves some of the denser albumen already present, and so brings about the dilution of the latter in some degree. At the same time, by this process of diffusion, the fluid layer is rendered more dense, coming finally to the consistency of the thin layer of the laid egg. The thin albumen *layer*, however, does not owe its existence in any sense to this dilution factor, but to a definite secretion of a thin albumen by the glands of the isthmus and uterus.

6. The addition of albumen to the egg is completed only after it has been in the uterus from five to seven hours.

7. Before the acquisition of albumen by the egg is completed a fairly considerable amount of shell substance has been deposited on the shell membranes.

8. For the completion of the shell and the laying of the egg from twelve to sixteen, or exceptionally even more, hours are required.

## APPENDIX

The following table gives *in extenso* the original data on which this paper is based. It should be said that the weights are in grams.



TABLE A  
*Data on eggs*

BIRD NUMBER	DATE OF LAYING	HOUR OF LAYING	LOCATION AND CONDITION OF EGG	WEIGHT OF EGG	WEIGHT OF SHELL AND MEMBRANES	WEIGHT OF YOLK	WEIGHT OF ALBUMEN	RATIO OF YOLK TO ALBUMEN	WEIGHT OF WATER IN ALBUMEN	PER CENT OF ALBUMEN	WEIGHT OF NITROGEN IN ALBUMEN	PER CENT OF NITROGEN IN ALBUMEN
8017	1910 March 20	2 P.M.	Normal, laid	48.29	5.34	15.59	27.36	1:1.755				
	March 22	9 A.M.	Normal, laid	49.37	5.93	15.69	27.75	1:1.769				
	March 23	1 P.M.	Normal, laid	49.55	5.36	16.02	28.17	1:1.758				
	March 26	2 P.M.	Normal, laid	50.17	5.49	15.93	28.73	1:1.805				
	March 28	2:30 P.M.	Normal, laid	50.85	5.99	15.95	28.90	1:1.811				
	March 30	8:30 A.M.	Normal, laid	49.02	6.23	16.40	26.39	1:1.609				
			Means of normal, laid eggs	49.54	5.72	15.93	27.89	1:1.750				
8009	March 30	12 M.	In albumen portion, 11 cms. in front of cranial end of isthmus	24.03	0	14.50	9.53	1:0.657				
	1910 March 20	11 A.M.	Normal, laid	55.78	5.93	16.64	33.21	1:1.996				
	March 21	4 P.M.	Normal, laid	54.78	6.16	16.34	32.28	1:1.976				
	March 23	9 A.M.	Normal, laid	62.07	6.71	17.35	38.01	1:2.191				
	March 24	10:30 A.M.	Normal, laid	57.66	6.12	16.83	34.71	1:2.062				
			Means of normal, laid eggs	57.57	6.23	16.79	34.55	1:2.058				
	March 24	1:45 P.M.	At caudal end of albumen portion. No membrane	27.20	0	15.38	11.82	1:0.769				
8005	1910 March 3	1 P.M.	Normal, laid	52.57	5.94	15.66	30.97	1:1.978				
	March 4	4 P.M.	Normal, laid	52.99	5.93	15.71	31.35	1:1.996				
	March 7	10 A.M.	Normal, laid	53.46	6.13	15.83	31.50	1:1.990				

TABLE A (continued)

8005	1910	March 8	9 A.M.	Normal, laid	53.66	5.91	15.44	32.31	1:2.093	
		March 9	8.30 A.M.	Normal, laid	52.91	5.65	15.94	31.32	1:1.985	
		March 10	9 A.M.	Normal, laid	52.54	5.81	15.78	30.95	1:1.961	
		March 11	9 A.M.	Normal, laid	52.48	5.62	15.78	31.08	1:1.970	
		March 12	9 A.M.	Normal, laid	51.42	5.49	15.43	30.50	1:1.977	0.6036 1.98
1293		March 13	8.30 A.M.	Normal, laid	53.22	5.62	15.48	32.12	1:2.075	0.6387 1.973
				Means of normal, laid eggs	52.81	5.79	15.67	31.34	1:2.000	0.6188 1.977
		March 13	11.30 A.M.	At caudal end of albumen portion. No membrane	29.53	0	15.87	13.66	1:0.861	
	1909	April 15		Normal, laid	55.5	9.00	16.00	30.50	1:1.906	
		April 16		Normal, laid	57.0	9.00	16.50	31.50	1:1.900	0.5635 4.125
266	1911	August 2	9.45 A.M.	Means of normal, laid eggs	56.25	9.00	16.25	31.00	1:1.908	
				Just entering isthmus. Little cap of membrane on caudal tip	29.5	0	16.00	13.50	1:0.844	
				Normal laid, Entering isthmus. Covered with membrane except for a little of cranial tip	51.00	6.00	14.00	31.00	1:2.214	
					31.00		14.50	16.50	1:1.138	
				Normal, laid	55.02	5.09	17.49	32.44	1:1.855	
8027		April 10	1 P.M.	Normal, laid	55.65	6.15	17.55	31.95	1:1.821	0.6374 1.995
		April 11	3.30 P.M.	Normal, laid	53.34	5.79	16.05	31.50	1:1.963	0.6387 2.028
		April 13	8.30 A.M.	Normal, laid	54.75	5.99	16.37	32.39	1:1.979	0.6381 1.970

TABLE A (continued)

BIRD NUMBER	DATE OF LAYING	HOUR OF LAYING	LOCATION AND CONDITION OF EGG	WEIGHT OF EGG	WEIGHT OF SHELL AND MEMBRANES	WEIGHT OF YOLK	WEIGHT OF ALBUMEN	RATIO OF YOLK TO ALBUMEN	WEIGHT OF WATER IN ALBUMEN	PER CENT OF WATER IN ALBUMEN	WEIGHT OF ALBUMEN	PER CENT OF NITROGEN IN ALBUMEN
8027	1911 April 13	12.30 P.M.	Means of normal, laid eggs	54.69	5.76	16.87	32.07	1 : 1.902			0.6381	1.998
			Entering isthmus. Covered with membrane except for a little of cranial tip	32.44	0.24	15.69	16.51	1 : 1.052			0.6233	3.775
108	1910 August 2	10 A.M. 1 P.M.	Normal, laid	61.53			38.4895		33.6536	87.440	6800	1.78
			Entering isthmus. Covered with membrane except for a little of cranial tip	36.33			21.4015		17.0036	79.450	6515	3.04
2554	1910 April 4 April 6		Normal, laid	59.5	9.0	19.0	31.5	1 : 1.638				
			Normal, laid	61.5	8.5	20.0	33.0	1 : 1.650				
	April 6		Means of normal, laid eggs	60.5	8.75	19.5	32.25	1 : 1.654				
			In upper part of isthmus. Membrane complete but thin	32.0		16.5	15.5	1 : 0.939				
8008	1911 March 24 March 26 March 27 March 29	4 P.M. 9 A.M. 1.30 P.M. 11 A.M.	Normal, laid	47.43	5.27	12.27	29.89	1 : 2.436				
			Normal, laid	52.42	5.79	13.89	32.74	1 : 2.357				
			Normal, laid	50.35	5.52	13.25	31.58	1 : 2.383				
			Normal, laid	51.27	5.87	13.93	31.47	1 : 2.259			0.6483	2.060

TABLE A (continued)

8008	1911 March 30	11 A.M.	Normal, laid	49.53	5.25	13.62	30.66	1 : 2.251	0.03312.065
			Means of normal, laid eggs	50.20	5.54	13.39	31.27	1 : 2.335	0.64072.0625
	March 30	3 P.M.	In upper part of isthmus. Membrane thin	28.33	0.28	12.57	15.48	1 : 1.232	0.53723.47
1367	1911 March 25	10.30 A.M.	Normal, laid	48.71	5.12	16.11	27.48	1 : 1.706	
	March 26	11 A.M.	Normal, laid	48.73	5.44	15.95	27.34	1 : 1.714	
	March 27	1.30 P.M.	Normal, laid	47.11	5.13	15.56	26.42	1 : 1.698	
	March 28	12.30 P.M.	Normal, laid	47.90	5.52	16.03	26.35	1 : 1.644	
	March 29	1 P.M.	Normal, laid	48.18	5.37	15.90	26.71	1 : 1.680	
	March 30	4 P.M.	Normal, laid	47.53	5.34	15.27	26.92	1 : 1.703	
	March 31	5 P.M.	Normal, laid	47.40	5.31	14.65	27.44	1 : 1.873	
	April 2	7.30 A.M.	Normal, laid	50.40	5.77	15.89	28.74	1 : 1.809	
	April 3	9 A.M.	Normal, laid	50.05	5.41	15.97	28.67	1 : 1.795	
			Means of normal, laid eggs	48.45	5.40	15.70	27.34	1 : 1.741	
416	April 3	1.30 P.M.	Three cm. below beginning of isthmus. Membrane thin	32.08	0.39	16.12	15.57	1 : 0.966	
	1910 April 15		Normal, laid	47.0	6.5	14.5	26.0	1 : 1.793	
	April 20		Normal, laid	48.0	7.5	15.0	25.5	1 : 1.700	
			Means of normal, laid eggs	47.5	7.0	14.75	25.75	1 : 1.746	
	April 22		Two cm. above caudal end of isthmus	31.0	2.0	16.0	13.0	1 : 0.813	
	1911 March 3	1 P.M.	Normal, laid	50.92	5.78	15.97	29.17	1 : 1.827	
8010	March 4	4 P.M.	Normal, laid	50.35	5.40	15.87	29.08	1 : 1.832	
	March 6	9 A.M.	Normal, laid	52.49	5.85	16.08	30.56	1 : 1.901	

TABLE A (continued)

BIRD NUMBER	DATE OF LAY- ING	HOOR OF LAY- ING	LOCATION AND CONDITION OF EGG	WEIGHT OF EGG	WEIGHT OF ARTERIAL AND MEMBRANES	WEIGHT OF YOLK	WEIGHT OF ALBUMEN	RATIO OF YOLK TO ALBUMEN	WEIGHT OF WATER IN ALBUMEN	PER CENT OF WATER IN ALBUMEN	WEIGHT OF ALBUMEN	PER CENT OF NITROGEN IN ALBUMEN
8010	1911 March 8	1 P.M.	Normal, laid	50.73	5.59	16.47	28.67	1 : 1.741				
	March 9	4 P.M.	Normal, laid	51.92	5.71	16.20	30.01	1 : 1.852				
	March 11	9 A.M.	Normal, laid	51.98	5.98	15.30	30.70	1 : 2.007				
	March 12	10 A.M.	Normal, laid	51.73	5.99	15.67	30.07	1 : 1.919				
	March 13	11 A.M.	Normal, laid	50.43	5.57	14.81	30.05	1 : 2.029				
	March 14	4 P.M.	Normal, laid	50.16	5.63	14.48	30.05	1 : 2.075				
	March 16	9 A.M.	Normal, laid	52.13	6.04	15.64	30.45	1 : 1.947				
	March 17	10 A.M.	Normal, laid	54.11	5.88	16.41	31.82	1 : 1.939				
			Means of normal, laid eggs	51.54	5.77	15.72	30.06	1 : 1.912				0.6075 1.995
				30.00	0.53	14.04	15.43	1 : 1.099				0.5712 1.795
8018	1911 March 17	1.30 P.M.	In lower part of isthmus	59.20	6.69	17.52	34.99	1 : 1.997				0.5894 1.895
	May 20	2 P.M.	Normal, laid	59.25	6.45	17.21	35.59	1 : 2.068				0.5092 3.300
	May 21	4 P.M.	Normal, laid	62.26	6.79	17.86	37.61	1 : 2.106				
	May 23	9 A.M.	Normal, laid	59.70	6.59	17.10	36.01	1 : 2.106				0.7240 1.925
	May 24	10.30 A.M.	Normal, laid	60.10	6.63	17.42	36.05	1 : 2.069				0.6680 1.855
			Means of normal, laid eggs	37.27	0.58	16.37	20.32	1 : 1.241				0.6960 1.890
	May 24	2.10 P.M.	In lower part of isthmus	46.0	8.5	14.5	23.0	1 : 1.586				0.6756 3.325
			Normal, laid	33.0	2.0	14.5	16.5	1 : 1.138				
324	1910 July 19		In uterus. No shell formed	56.04								
	July 22											
117	1910 August 1	10.30 A.M.	Normal, laid	36.15								
	August 1	3.30 P.M.	In uterus, but no visible shell formed									
									30.1016	87.460	61.541	79
									14.9943	79.580	57.118	03

TABLE A (continued)

8038	1911	March 24	10 A.M.	Normal, laid	61.11	6.82	18.16	36.13	1 : 1.990	
		March 25	1 P.M.	Normal, laid	60.98	6.59	17.81	36.58	1 : 2.054	
		March 26	4 P.M.	Normal, laid	59.90	6.58	17.51	35.81	1 : 2.045	0.63561.775
		March 28	9 A.M.	Normal, laid	61.69	7.06	17.60	37.03	1 : 2.104	0.66281.790
				Means of normal, laid eggs	60.92	6.76	17.77	36.39	1 : 2.048	0.64921.783
8039		March 28	4.15 P.M.	In uterus, but no shell formed. Egg surrounded by fluid in uterus	43.93	0.76	17.30	25.87	1 : 1.495	0.71792.775
	1911	March 3	1 P.M.	Normal, laid	56.99	6.03	16.54	34.42	1 : 2.081	
		March 5	9.30 A.M.	Normal, laid	60.83	6.39	17.87	36.57	1 : 2.046	
		March 6	11 A.M.	Normal, laid	58.96	6.07	17.51	35.38	1 : 2.021	
		March 7	1 P.M.	Normal, laid	58.39	6.01	17.26	35.12	1 : 2.035	
8030		March 8	4 P.M.	Normal, laid	60.36	6.34	17.37	36.65	1 : 2.110	0.63511.733
		March 10	9 A.M.	Normal, laid	59.80	6.49	17.48	35.83	1 : 2.050	0.66181.847
				Means of normal, laid eggs	59.22	6.22	17.34	35.66	1 : 2.057	0.64851.790
		March 10	1.30 P.M.	In uterus, but no visible shell	45.15	0.96	17.25	26.94	1 : 1.562	0.67272.497
	1911	April 10	1 P.M.	Normal, laid	58.50	6.63	18.94	32.93	1 : 1.739	0.54661.66
8033		April 12	8.30 A.M.	Normal, laid	59.48	7.19	19.14	33.15	1 : 1.732	0.50021.69
				Means of normal, laid eggs	58.99	6.91	19.04	33.04	1 : 1.735	0.55341.675
		April 12	2.15 P.M.	In uterus but no visible shell formed. Some fluid in uterus	46.67	0.88	19.13	26.66	1 : 1.394	0.61182.295

TABLE A (continued)

BIRD NUMBER	DATE OF LAYING	HOUR OF LAYING	LOCATION AND CONDITION OF EGG	WEIGHT OF EGG	WEIGHT OF SHELL AND MEMBRANES	WEIGHT OF YOLK	WEIGHT OF ALBUMEN	RATIO OF YOLK TO ALBUMEN	WEIGHT OF WATER IN ALBUMEN	PER CENT OF WATER IN ALBUMEN	WEIGHT OF NITROGEN IN ALBUMEN	PER CENT OF NITROGEN IN ALBUMEN
200	1910 August 3	8:30 A.M.	Normal, laid	48.16			27.608		23.9627	86.800	51971.88	
			In uterus, small amount of shell	37.98			21.371		17.7154	82.890	53712.51	
1061	1910 June 10 June 11		Normal, laid	53.0	7.0	16.5	29.5	1:1.788				
			Normal, laid	51.0	8.0	15.0	28.0	1:1.867				
			Means of normal, laid eggs	52.0	7.5	15.75	28.75	1:1.825				
			In uterus, small amount of shell formed	45.0	4.5	15.5	25.0	1:1.613				
387	1910 July 26	9 A.M. 3:30 P.M.	Normal, laid	56.25			34.9305		30.7005	87.890	59971.72	
			In uterus, small amount of shell	46.50			29.9665		25.9559	86.620	57411.92	
			Normal, laid	49.07	5.37	15.73	27.97	1:1.778				
8021	1911 March 24 March 25 March 28	9 A.M. 10:30 A.M. 8:30 A.M.	Normal, laid	47.83	4.86	15.05	27.92	1:1.855			0.5444	1.95
			Normal, laid	50.89	5.30	15.91	29.68	1:1.865			0.5802	1.975
			Means of normal, laid eggs	49.28	5.18	15.56	28.52	1:1.833			0.5653	1.96
			In uterus. Some shell formed	43.66	1.35	15.18	27.13	1:1.787			0.5616	2.07

# COMPARATIVE STUDIES ON THE EFFECTS OF ALCOHOL, NICOTINE, TOBACCO SMOKE AND CAFFEINE ON WHITE MICE

## I. EFFECTS ON REPRODUCTION AND GROWTH

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ONE FIGURE

## CONTENTS

Introduction.....	133
Alcohol.....	133
Nicotine and tobacco smoke.....	135
Caffeine.....	135
Methods.....	135
Effects on the weight and health of the adult mice.....	138
Effects on the fecundity of the adults and viability of the young.....	139
Growth of the young subjected to the same conditions as their parents.....	139
Growth of the young not subjected to the drugs.....	145
Comparison of the growth of mice given drugs with those not given drugs.....	146
Summary.....	147
Bibliography.....	149

## INTRODUCTION

### *Alcohol*

The effects of alcohol on animal offspring has been shown in several investigations to be injurious. Laitinen (31) found that alcoholized rabbits and guinea pigs had more stillborn young than the controls; and that the growth of the living young was retarded. Of 23 pups from a pair of alcoholic dogs in Hodge's (28) experiments, 8 were deformed, 9 were born dead, and only 4 were viable. From the control pair 4 were deformed, none were born dead and 41 were viable. Forty-three per cent of the eggs



of Ceni's (10) alcoholized fowls developed normally under ordinary conditions in comparison to 77 per cent of the controls. A fluctuating temperature at the beginning of incubation prevented all alcoholic eggs from developing perfectly while 27 per cent of the control eggs were normal.

Todde (55) alcoholized 15 roosters from two to four months and concluded that alcohol causes a torpidity of the testicles, acting probably through its affinity for the central nervous system. Bertholet (5) found in 37 out of 39 cases of alcoholism in men more or less of an atrophy of the testicles. Ceni's (10) three alcoholized hens laid less than half the average number of eggs, but since fowls are abnormally developed in this respect we might anticipate that anything which injured their general health would disturb the laying function without drawing the conclusion that in other animals alcohol would promote sterility. Laitinen (31 and 33) found that alcoholized rabbits and guinea pigs had more young than the controls. The investigations of the Eugenics Laboratory (4, 18 and 43) and of Laitinen (33) demonstrate that more children are born to alcoholic than to sober parents.

As to the effect of alcohol on human offspring, Demme (16) compared 10 non-alcoholic with 10 alcoholic families and found that 82 per cent of the children of the former and only 17.5 per cent of the latter were normal. Sollier (53) states that it is demonstrated that progeny conceived during drunkenness is doomed to idiocy. Morel (41) gives the history of an alcoholic family through four generations to final extinction, concluding that alcoholic intoxication produces degeneracy, depravity and idiocy. On the other hand Pearson, Elderton and Barrington (43, 18 and 4) believe that their statistical data prove that extreme alcoholism is a result, not a cause of degeneracy and that the abnormal pedigrees are due to defective stock and not to alcohol. In an investigation of about four thousand school children of alcoholic and sober parents they could not find that parental alcoholism had any unfavorable effect on the offspring.

*Nicotine and tobacco smoke*

Tobacco infusion injected into young rabbits by Richon and Perrin (46) had a decided stunting effect; however, if the injections were stopped, the animals gained rapidly and soon equalled the controls. Rabbits subjected to the fumes of tobacco by Zebrowski (60) for six to eight hours daily for several months suffered from loss of appetite and great emaciation, losing from 20 to 47 per cent of their weight. Nicotine injected into eggs by Féré (19) seemed to be a poison to some and a stimulant to others. Fleig (21) subjected guinea pigs to heavy inhalations of tobacco smoke, to injections of extract of smoke or injections of nicotine, and obtained abortions, still-births, under-sized, weak and stunted young. Unfortunately he fails to give definite details and conditions of his experiments.

Thirteen cases of sexual impotence caused by the abuse of tobacco are reported by Cannata (9); abstinence from smoking restored the men to a normal condition. Hitchcock (52) at Amherst College and Seaver (52) at Yale found by measuring students that the smokers developed less in height and lung capacity during their college course than the nonsmokers. Meylan (39), however, at Columbia could not find that smoking had any decided effect on growth.

*Caffeine*

No experiments could be found on the effects of caffeine on growth and reproduction. Rivers and Weber (47) show that caffeine increases capacity for muscular work. The investigations of Crämer (12), Pincussohn (44), Sasaki (50), Schulzenstein (51) and Voit (57) indicate that coffee and tea retard digestion.

## METHODS

A comparative study was undertaken to test the effects of alcohol, nicotine and caffeine on the offspring of animals, when fed in small enough amounts so as not to injure their health. White mice were chosen for the experiments since they breed rapidly, so that the results could be based on large numbers, and a

second generation could be quickly obtained to compare with the first.

The experiments were started with 30 female and 15 male white mice about four months old. They were bought from a dealer and guaranteed not to be brothers and sisters. Four hundred and forty-one young were born of the first generation in seven months. Twenty females and 12 males of this number were bred at the age of two and a half months, care being taken to prevent inbreeding. These had 230 young in four months. Five lines were carried; one was given alcohol, another nicotine, a third was subjected to the fumes of tobacco smoke, a fourth received caffeine and the fifth line was carried for controls:

Two females and one male were kept in a cage; each female was moved to a separate cage before her young were born and remained there as long as they were suckling, which lasted from twenty-five to thirty days. The cages were of wire mesh 6 inches wide, 5 inches deep and 12 inches long.

The regular diet of all the mice was buckwheat, and crackers soaked in milk; every few days they were given carrots, grass, meat, etc.

#### *The alcohol line*

The first generation in this line consisted of 6 females and 3 males, and in the second generation, the offspring of the first, of 5 females and 3 males.

Two cubic centimeters per mouse of 35 per cent alcohol were added daily to the crackers and milk of these mice. Instead of water they drank 35 per cent alcohol which was placed in bottles containing siphons; so the animals drank directly from the bottles, and evaporation was prevented. The mice were first given 10 per cent alcohol which was gradually increased to 45 per cent. Thirty-five per cent was found to be a safe medium since stronger percentages sometimes intoxicated the mice.

All the mice in this line except 16 received alcohol, the young beginning to take it at the age of three weeks. Sixteen young were given no alcohol themselves although their mothers received it for sixteen days after the birth of the young.

*The nicotine line*

The first generation in this line consisted of 6 females and 3 males, and the second generation, the offspring of the first, of 3 females and 2 males.

This line had 2 cc. per mouse of 1:1000 nicotine solution added to their crackers and milk daily, and the same solution was substituted for drinking water in bottles with siphons. Different strengths of nicotine had been tested on white mice from 1:2000 to 1:500 solution. The last was fatal but on 1:1000 they remained in good health, although this solution was found to kill grey rats in nine days.

All the mice in this line except 6 received nicotine, the young beginning to take it at the age of three weeks. Six young were given no nicotine themselves although their mothers received it for sixteen days after the birth of the young.

*The line subjected to the fumes of tobacco*

The first generation in this line consisted of 6 females and 3 males, and the second generation, the offspring of the first, of 8 females and 4 males.

These mice were subjected to tobacco smoke for about five minutes at a time and then aired for five or ten minutes; this was repeated for two hours each day. The smoke chamber was a bell jar made air tight by being placed on glass and moistened around the bottom. About 4 grams of Connecticut leaf tobacco were burned each day in a clay pipe inserted in a rubber cork in the top of the bell jar. The smoke was drawn through the jar by an air pump, the tube for this purpose being in the rubber cork.

All the mice of this line except 9 were subjected to the fumes of tobacco, the young being put in the smoke chamber from the time they were a few days old. Nine young were not subjected to the fumes of tobacco, although their mothers were each day while they were suckling.

*The caffeine line*

This line consisted of 6 female and 3 male mice. No second generation was obtained for breeding since many of the young died and others were eaten by their parents.

Each mouse had 2 cc. of 1:100 caffeine citrate solution added to his crackers and milk daily, and drank this solution instead of water. The caffeine citrate was first given in a 1:500 solution which was gradually increased to a 1:50 solution. Since they did not seem to be thriving on this strength it was decreased to 1:100, on which they remained in good health.

All the mice in this line except 8 received caffeine, the young beginning to take it at the age of three weeks. Eight young were given no caffeine themselves, although their mothers received it for sixteen days after the birth of the young.

*The control line*

The first generation of control mice consisted of 6 females and 3 males. Since these mice had few young and many were eaten there were none of the second generation ready for breeding three months after the experiments were started. Therefore 6 young females and 3 males were obtained from the same source as the first lot of mice so as to serve as controls for comparison with the second generations of the other lines.

## EFFECTS ON THE WEIGHT AND HEALTH OF THE ADULT MICE

The females were weighed each time after they had given birth to a litter, and the males from time to time. During the course of the experiments all the mice gained in weight; in the first generation, carried seven months, the alcohol mice gained 6 grams each on an average, and the others gained 2 grams; in the second generation carried four months, the alcohol mice gained 2 grams and the others 1 gram. This would indicate that the mice remained healthy and that alcohol has a fattening effect.

## EFFECTS ON FECUNDITY OF THE ADULTS AND VIABILITY OF THE YOUNG

A record was kept of the litters of each female. Many of the young in all the lines were eaten. In the tables 1 to 13 only those young that died from lack of vitality are recorded.

TABLE 1

*Record of the young of each female**Control line. First generation*

FEMALE	NUMBER OF MONTHS OBSERVED	NUMBER OF LITTERS	NUMBER OF YOUNG BORN	NUMBER OF YOUNG THAT DIED
A.....	7	3	18	0
B.....	7	2	12	0
C.....	7	2	16	0
D.....	7	2	7	0
E*.....	3	1	7	0
F†.....				
Total.....	4	10	60	0
	3			

\* Female E died at the end of three months. No pathological condition could be found.

† Female F was killed by accident at the beginning of the experiments.

TABLE 2

*Alcohol line. First generation*

FEMALE	NUMBER OF MONTHS OBSERVED	NUMBER OF LITTERS	NUMBER OF YOUNG BORN	NUMBER OF YOUNG THAT DIED
A.....	7	2	17	1
B.....	7	2	12	1
C.....	7	4	23	1
D.....	7	4	15	2
E.....	7	2	14	4
F*.....				
Total.....	5	14	81	9
	1	3½ wks.		

\* Female F died three and a half weeks after the experiments were started. No pathological conditions could be found.

TABLE 3

*Nicotine line. First generation*

FEMALE	NUMBER OF MONTHS OBSERVED	NUMBER OF LITTERS	NUMBER OF YOUNG BORN	NUMBER OF YOUNG THAT DIED
A.....	7	3	13	1
B.....	7	2	7	0
C.....	7	1	4	4
D.....	7	2	13	2
E.....	7	5	36	2
F.....	7	4	31	9
Total.....6	7	17	104	18

TABLE 4

*Line subjected to tobacco fumes. First generation*

FEMALE	NUMBER OF MONTHS OBSERVED	NUMBER OF LITTERS	NUMBER OF YOUNG BORN	NUMBER OF YOUNG THAT DIED
A.....	7	4	23	9
B.....	7	4	30	5
C.....	7	3	22	6
D.....	7	4	21	7
E*.....	4	3 <sup>†</sup>	15	14
F†.....				
Total.....4	7	18	111	41
1	4			

\* E was killed by accident at the end of three months.

† F died at the beginning of the experiments.

‡ One of these litters was an abortion.

TABLE 5

*Caffeine line. First generation*

FEMALE	NUMBER OF MONTHS OBSERVED	NUMBER OF LITTERS	NUMBER OF YOUNG BORN	NUMBER OF YOUNG THAT DIED
A.....	7	3	15	0
B.....	7	3	16	8
C.....	7	2	11	5
D.....	7	3	27	2
E.....	7	2	7	4
F.....	7	3	9	3
Total.....6	7	16	85	22

TABLE 6

*Control line. Second set*

FEMALE	NUMBER OF MONTHS OBSERVED	NUMBER OF LITTERS	NUMBER OF YOUNG BORN	NUMBER OF YOUNG THAT DIED
A.....	4	1	7	0
B.....	4	2	12	0
C.....	4	2	9	0
D.....	4	2	9	0
E.....	4	1	2	0
F.....	4	1	4	0
Total.....6	4	9	43	0

TABLE 7

*Alcohol line. Second generation*

FEMALE	NUMBER OF MONTHS OBSERVED	NUMBER OF LITTERS	NUMBER OF YOUNG BORN	NUMBER OF YOUNG THAT DIED
A.....	4	1	5	0
B.....	4	2	13	1
C.....	4	2	16	2
D.....	4	2	13	3
E.....	2	1	9	1
Total.....4	4	8	56	7
1	2			

TABLE 8

*Nicotine line. Second generation*

FEMALE	NUMBER OF MONTHS OBSERVED	NUMBER OF LITTERS	NUMBER OF YOUNG BORN	NUMBER OF YOUNG THAT DIED
A.....	4	2	11	5
B.....	4	2	13	0
C.....	4	1	6	0
Total.....4	4	5	30	5



TABLE 9

*Line subjected to tobacco fumes. Second generation*

FEMALE	NUMBER OF MONTHS OBSERVED	NUMBER OF LITTERS	NUMBER OF YOUNG BORN	NUMBER OF YOUNG THAT DIED
A.....	4	3	21	3
B.....	4	3	15	1
C.....	4	2	11	2
D.....	4	4	22	9
E.....	4	2	12	3
F.....	4	1	8	1
G.....	3	1	6	1
H.....	3	1	6	6
Total.....	6 2	4 3	17	101 26

TABLE 10

*Record of the young of the first generation  
Summary of tables 1, 2, 3, 4 and 5*

LINE	NUMBER OF FEMALES	NUMBER OF MONTHS OBSERVED	NUMBER OF LITTERS	NUMBER OF YOUNG BORN	NUMBER OF YOUNG THAT DIED
Control.....	{ 4 1 }	{ 7 3 }	10	60	0
Alcohol.....	5	7	14	81	9
Nicotine.....	6	7	17	104	18
Smoked.....	{ 4 1 }	{ 7 4 }	18	111	41
Caffeine.....	6	7	16	85	22

TABLE 11

*Record of the young of the second generation  
Summary of tables 6, 7, 8 and 9*

LINE	NUMBER OF FEMALES	NUMBER OF MONTHS OBSERVED	NUMBER OF LITTERS	NUMBER OF YOUNG BORN	NUMBER OF YOUNG THAT DIED
Control.....	6	4	9	43	0
Alcohol.....	{ 4 1 }	{ 4 2 }	8	56	7
Nicotine.....	3	4	5	30	5
Smoked.....	{ 6 2 }	{ 4 3 }	17	101	26

TABLE 12

*Average of one female of the first generation for seven months*

LINE	AVERAGE NUMBER OF LITTERS	AVERAGE NUMBER OF YOUNG	PER CENT OF YOUNG THAT DIED
Control.....	2.2	13.3	0
Alcohol.....	2.8	16.1	11.1
Nicotine.....	2.8	17.3	17.3
Smoked.....	4.0	24.6	37.0
Caffeine.....	2.7	14.1	25.3

TABLE 13

*Average of one female of the second generation for four months*

LINE	AVERAGE NUMBER OF LITTERS	AVERAGE NUMBER OF YOUNG	PER CENT OF YOUNG THAT DIED
Control.....	1.5	7.1	0
Alcohol.....	1.8	12.4	12.5
Nicotine.....	1.66	10.0	16.6
Smoked.....	2.26	13.4	26.0

In both generations the mice subjected to tobacco fumes had more young than any of the other lines, whereas the controls had the fewest young. Tobacco fumes had a marked effect on the viability of the young, since 37 per cent of the first generation died from lack of vitality and 26 per cent of the second. One abortion occurred in this line. Caffeine was also injurious to the young, 25 per cent dying. Nicotine and alcohol had a less noticeable influence. None of the control young died in either generation.

#### GROWTH OF THE YOUNG SUBJECTED TO THE SAME CONDITIONS AS THEIR PARENTS

The young were either weighed singly or in litters at birth and every week for eight weeks. Since it was not always possible to weigh them before they suckled, the variations in the birth weights have little significance. The records were not carried beyond eight weeks for the young females often become pregnant at that time.

TABLE 14

*Weekly growth of the young of the first generation subjected to the same conditions as their parents*

LINE	AVERAGE WEIGHT OF FEMALE PARENTS	AVERAGE WEIGHT OF MALE PARENTS	NUMBER YOUNG WEIGHED	AVERAGE WEIGHT AT BIRTH PER MUSE	AVERAGE WEIGHT AT ONE WEEK	AVERAGE WEIGHT AT TWO WEEKS	AVERAGE WEIGHT AT THREE WEEKS	AVERAGE WEIGHT AT FOUR WEEKS	AVERAGE WEIGHT AT FIVE WEEKS	AVERAGE WEIGHT AT SIX WEEKS	AVERAGE WEIGHT AT SEVEN WEEKS	AVERAGE WEIGHT AT EIGHT WEEKS
	grams	grams		grams	gm.	gm.	gm.	grams	grams	grams	grams	grams
Control.....	24.8	20.7	17	1.24	2.74	05.66	9.10	10.1	14.6	15.7	16.8	
Alcohol.....	30.6	24.5	20	1.30	3.15	06.49	7.12	12.0	14.0	16.4	17.5	
Nicotine.....	27.4	22.2	32	1.35	3.14	25.58	0.10	5.13	13.0	12.5	15.1	
Smoked.....	23.9	23.6	31	1.23	2.94	06.08	2.10	5.13	13.3	14.9	15.3	
Caffeine.....	27.9	22.2	27	1.30	3.04	25.37	7.10	10.7	10.7	12.1	13.9	

TABLE 15

*Weekly growth of the young of the second generation subjected to the same conditions as their parents*

LINE	AVERAGE WEIGHT OF FEMALE PARENTS	AVERAGE WEIGHT OF MALE PARENTS	NUMBER YOUNG WEIGHED	AVERAGE WEIGHT AT BIRTH PER MUSE	AVERAGE WEIGHT AT ONE WEEK	AVERAGE WEIGHT AT TWO WEEKS	AVERAGE WEIGHT AT THREE WEEKS	AVERAGE WEIGHT AT FOUR WEEKS	AVERAGE WEIGHT AT FIVE WEEKS	AVERAGE WEIGHT AT SIX WEEKS	AVERAGE WEIGHT AT SEVEN WEEKS
	grams	gm.		grams	gm.	gm.	grams	grams	grams	grams	grams
Control.....	29.5	25.14	1.47	3.04	0.6.9	7.1	9.9	10.9	14.0		
Alcohol.....	23.3	22.13	1.35	2.84	9.6.75	9.6	12.2	14.0	16.0		
Nicotine.....	20.5	23.10	1.3	2.94	5.5.5	8.00	10.5	13.0	15.0		
Smoked.....	20.0	20.26	1.2	2.44	5.5.3	7.5	10.5	12.7	13.8		

One hundred and thirty-three young mice of the first generation and 63 of the second were subjected to the same conditions as their parents. Although there was much variation in the same line or even in the same litter, on the whole the five lines in both generations grew at about the same rate. The alcohol young excelled all the others.

There seems to be no constant correlation between the weight of the parent and growth of the young. For instance the alcohol adults are large in the first generation and the controls are small,

while the reverse is true of the second generation; yet the alcohol young are larger in both generations. The caffeine adults are large but their young are the smallest of all.

#### GROWTH OF THE YOUNG NOT SUBJECTED TO THE DRUGS

The following Table (Table 16) shows the growth of the offspring of drugged parents, not themselves subjected to the drugs:

TABLE 16

*Growth of the young not subjected to the drugs*

LINE	NUMBER YOUNG WEIGHED	AVERAGE WEIGHT AT BIRTH PER MOTHER	AVERAGE WEIGHT AT ONE WEEK	AVERAGE WEIGHT AT TWO WEEKS	AVERAGE WEIGHT AT THREE WEEKS	AVERAGE WEIGHT AT FOUR WEEKS	AVERAGE WEIGHT AT FIVE WEEKS	AVERAGE WEIGHT AT SIX WEEKS	AVERAGE WEIGHT AT SEVEN WEEKS	AVERAGE WEIGHT AT EIGHT WEEKS
		grams	grams	grams	grams	grams	grams	grams	grams	grams
Control*.....	33	1.35	2.85	4.0	6.2	7.0	10.0	12.7	14.8	16.8
Alcohol.....	16	1.5	3.4	5.2	7.3	11.4	14.7	15.6	17.6	19.2
Nicotine.....	6	1.5	2.3	4.7	6.0	8.5	10.9	13.4	16.0	18.0
Smoked.....	9	1.1	2.89	4.25	5.1	7.1	10.5	13.2	14.8	15.4
Caffeine.....	8	1.6	2.2	4.0	5.4	8.0	10.9	13.0	15.2	17.0

\* The weights of the controls are based on both sets of controls in tables 14 and 15.

From this it is seen that the young of the alcohol line decidedly excelled all the others in growth.

#### COMPARISON OF THE GROWTH OF MICE GIVEN DRUGS WITH THOSE NOT GIVEN DRUGS

The young of nicotine parents that were not given nicotine themselves excelled somewhat in weight those young that were given nicotine. Tobacco fumes had no appreciable effect, the smoked and non-smoked mice growing at about the same rate. The young of caffeine parents that were not given caffeine them-

TABLE 17

*Comparison of the growth of mice given drugs with those not given drugs*

MICE	NUMBER YOUNG WEIGHED	AVERAGE WEIGHT AT BIRTH PER MICE	AVERAGE WEIGHT AT ONE WEEK	AVERAGE WEIGHT AT TWO WEEKS	AVERAGE WEIGHT AT THREE WEEKS	AVERAGE WEIGHT AT FOUR WEEKS	AVERAGE WEIGHT AT FIVE WEEKS	AVERAGE WEIGHT AT SIX WEEKS	AVERAGE WEIGHT AT SEVEN WEEKS	AVERAGE WEIGHT AT EIGHT WEEKS
		grams	grams	grams	grams	grams	grams	grams	grams	grams
Control*.....	33	1.35	2.85	4.0	6.2	7.0	10.0	12.7	14.8	16.8
Without										
alcohol†.....	16	1.5	3.4	5.2	7.3	11.4	14.7	15.6	17.6	19.2
With alcohol*	39	1.32	3.3	4.97	6.5	9.6	12.1	14.0	16.2	17.5
Without										
nicotine†.....	6	1.5	2.3	4.7	6.0	8.5	10.9	13.4	16.0	18.0
With nicotine*	42	1.33	3.0	4.3	5.5	8.0	10.5	12.6	14.4	15.1
Non smoked†.....	9	1.1	2.89	4.25	5.1	7.1	10.5	13.2	14.8	15.4
Smoked*.....	57	1.22	2.7	4.2	5.7	8.0	10.5	13.0	14.4	15.3
Without										
caffeine†.....	8	1.6	2.2	4.0	5.4	8.0	10.9	13.0	15.2	17.0
With caffeine.....	27	1.3	3.0	4.2	5.3	7.7	10.1	10.7	12.1	13.9

\* These weights are the averages of the two generations from tables 14 and 15.

† These weights are from table 16. The parents were subjected to the drugs.

selves grew faster than those given caffeine. Although the young of the alcohol mice when given alcohol themselves excelled all the other mice in growth, other young of these same mice when not given alcohol grew even faster. The control mice grew faster than the caffeine mice, were excelled by the alcohol mice, but grew at about the same rate as the nicotine and smoked mice.

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## SUMMARY

1. The control mice had the fewest young of any of the lines; the fecundity of the alcohol, nicotine and caffeine mice was somewhat greater, while the mice subjected to tobacco fumes in both generations had almost twice as many young as the controls.

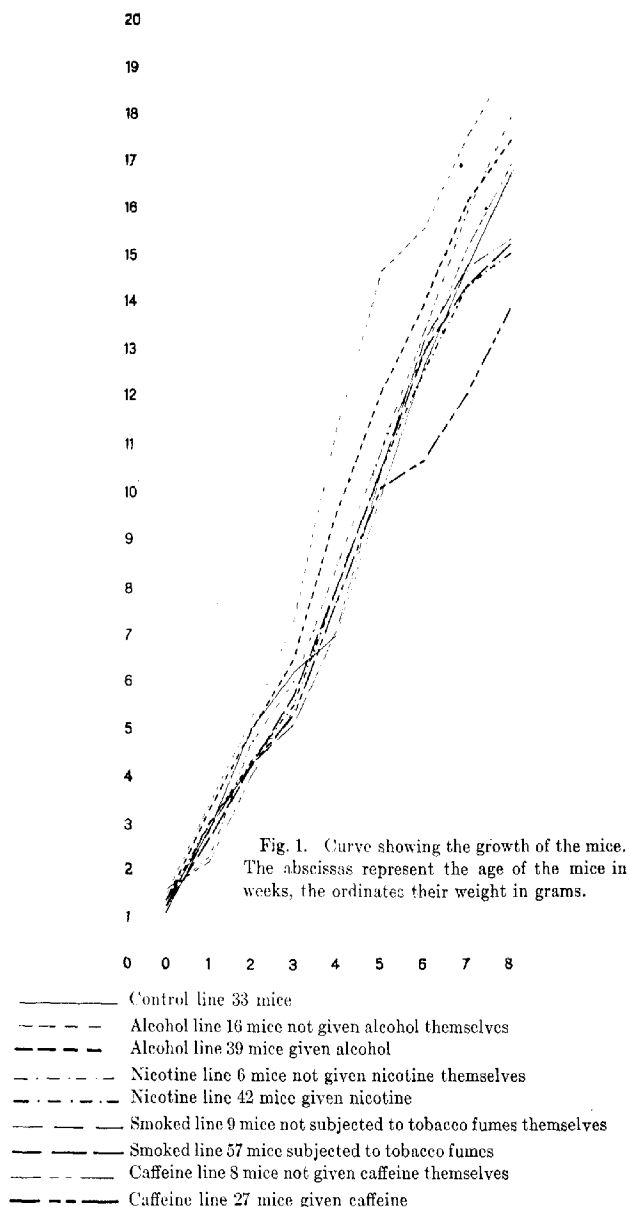
2. The mice subjected to tobacco fumes had the largest proportion of young that died from lack of vitality, 37 per cent dying in the first generation, and 26 per cent in the second. Caffeine also had an injurious effect, on the viability of the young, 25 per cent dying. Nicotine and alcohol had a less noticeable influence, 17.3 per cent and 11.1 per cent respectively died in the first generation, and 16.6 per cent and 12.5 per cent in the second. None of the control young died in either generation.

3. Out of 707 young born none were deformed, none were born dead, and only one abortion occurred. This took place in the smoke chamber.

4. When adult white mice were subjected to alcohol, nicotine, tobacco smoke and caffeine the growth of their offspring was not affected unfavorably.

5. When both adults and young were subjected to the drugs, caffeine had a slightly retarding influence on growth nicotine and tobacco smoke had no appreciable effect while the alcohol mice grew faster than the controls.

6. In all the experiments the young of the alcohol mice surpassed all the others in weight. They grew most rapidly when they themselves received no alcohol.



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BUFFALO, N. Y., January 1, 1912.

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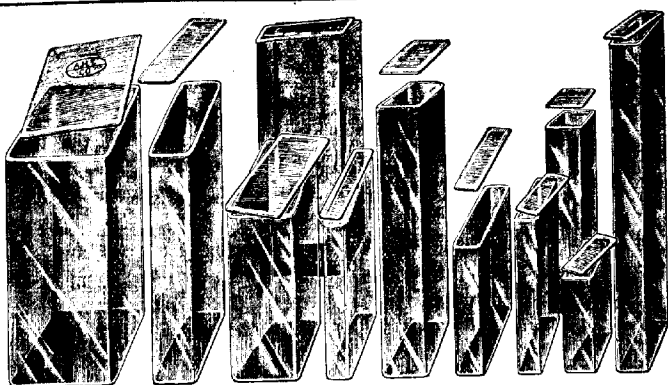
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14 x	14 x	3 "	22 x	7 x	3 "	42 x	10½ x	7½ "
15 x	9 x	7½ "	24 x	24 x	14 "	45 x	10 x	7 "
15 x	10½ x	5 "	26 x	6½ x	5 "	45 x	12 x	9 "
15 x	14 x	6 "	26 x	15 x	8 "	46 x	25 x	16 "
15 x	30 x	10 "	26 x	15 x	10 "	47 x	12 x	9 "
16 x	12 x	9 "	26 x	21 x	8 "	50 x	12 x	10 "
18 x	18 x	7 "	26 x	21 x	16 "	50 x	20 x	15 "
18 x	18 x	8 "	26 x	26 x	13 "	50 x	25 x	12 "
18 x	12 x	6 "	28 x	19 x	7 "	57 x	12 x	4 "
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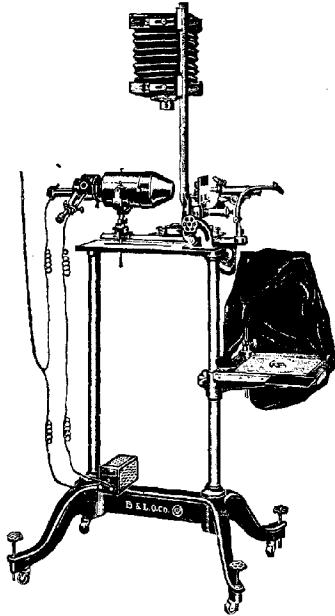
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## HEREDITY OF PIGMENTATION IN FUNDULUS HYBRIDS

FRANK W. BANCROFT

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THIRTY FIGURES

Ever since the rediscovery of Mendel's law, students of heredity who have investigated characters in the adult have habitually sought to determine whether or not these characters were inherited according to the Mendelian formula. Those, however, who have investigated the inheritance of larval and embryonal characters have usually not considered the facts from the Mendelian point of view but have sought to determine whether the inheritance was maternal or paternal. While it is possible that this point of view may be the most fruitful one from which to regard hybrids of distantly related species; still the work of Loeb, King, and Moore,<sup>1</sup> showed that for the larvae of closely related sea-urchins, at any rate, the Mendelian point of view could be adopted with profit. They found that in the larvae obtained by crossing *Strongylocentrotus purpuratus* and *S. franciscanus* certain characters of each species were dominant over the allelomorphic character in the other species, and the result was the same no matter whether the character in question was maternal or paternal. On account of the wealth of teleost material in Woods Hole and its favorable character for such investigations it was suggested by Dr. Loeb, whose constant helpfulness during the course of the work I wish to acknowledge, that I take up the study of inheritance in *Fundulus* from the Mendelian and the physiological points of view.

Previous work on Mendelian inheritance of this form is limited to the paper by Newman<sup>2</sup> who came to the conclusion that

<sup>1</sup> Arch. f. Entwickl.-mech. 1910, Bd. 29, p. 351.

<sup>2</sup> Jour. Exp. Zool., vol. 5, p. 503.



although some characters, such as the size and shape of the chromatophores, and the color pattern on head and body, show Mendelian dominance, that "nearly all of the characters observed may be classed as examples of blended inheritance of one sort or another."<sup>3</sup>

#### METHODS

Reciprocal crosses between *Fundulus heteroclitus* and *Fundulus majalis* were made in the usual way; both eggs and sperm from a number of individuals of each species being mixed for each experiment. The developing embryos were kept in Syracuse watch-glasses and fingerbowls, loosely covered with glass. The water was frequently changed and the eggs were carefully separated from the masses to insure a maximum supply of oxygen. After hatching the young fish were usually kept isolated in fingerbowls. They were fed with what they could pick off of foul eelgrass, and small pelagic organisms obtained by towing. Later fish flesh, flies, and liver were used for food. Some of the little fish were put out in the eel-pond in cloth cages of various kinds, but though they grew much faster than any that were kept in the laboratory so many of the cages got broken that only one of these fish was kept until the middle of September when they were transferred from Woods Hole to New York.

#### INHERITANCE OF COLOR CHARACTERS

Three kinds of chromatophores were observed, all three of them occurring in all the embryos of both pure species, and of both kinds of hybrids. They are:

1. Black opaque chromatophores. These are the first to appear, and apparently persist throughout the life of the fish.
2. Red opaque chromatophores, usually of a brick red or deep yellow color, showing white or creamy with a closed diaphragm. They sometimes take on a white or creamy color but can usually be easily distinguished from other tissues by their conspicuous white color when the light is turned off. They are nearly as

<sup>3</sup> Jour. Exp. Zool., vol. 5, p. 355.

large as the black chromatophores, appear in early embryonal life at about the same time as the black or but shortly after them, and disappear completely within a few days after hatching.

3. Small lemon yellow, or greenish yellow chromatophores. The pigment in these is transparent and it is only by carefully observing them in the most favorable locations such as the fins that the branched processes and the chromatophore-like shape of the cell can be made out. They first appear a few days before hatching and persist as long as the fish have been observed.

As all three kinds of chromatophores were found in all the embryos, and as no small differences in the color of the chromatophores that seemed to be significant were seen, the color differences observed were due to the variation of the chromatophores in (1) number, (2) size and shape, (3) location or arrangement, (4) rate of appearance and development.

Table I gives a summary of the main features of the pigmentation in the four forms studied.

An inspection of the table will show that in the first four characters (viz.: the red and black yolk chromatophores, head chromatophores, and red chromatophores of the lateral line) all of which are not concerned with the rate of development there is a well marked Mendelian dominance, while in the last two characters (viz.: the arrangement and time of appearance of the yolk chromatophores) both of which are concerned with the rate of development the dominance is not so evident. The characters will be considered *seriatim*.

#### 1. Red yolk chromatophores

The number of the red yolk chromatophores of the pure F. heteroclitus and both kinds of hybrids is about the same. No attempt was made to count them accurately but in all these three forms the red yolk chromatophores are very conspicuous features. In F. majalis, on the other hand, these chromatophores are so few that in one of the series they could not be found at all. In other series the first red yolk chromatophores appeared near the embryo from the fifth to the ninth day and increased slightly in

TABLE 1  
Principal color differences between pure forms and hybrids

PURE F. HETEROCLITUS	F. HETEROCLITUS ♀ X F. MAJALIS ♂ F. HETEROCLITUS EGG HYBRID	F. MAJALIS ♀ X F. HETEROCLITUS ♂ F. MAJALIS EGG HYBRID	PURE F. MAJALIS
1. Red yolk chromatophores large and abundant.	Red yolk chromatophores large and abundant, of same shape but smaller than in previous form.	Red yolk chromatophores large and abundant, but a little smaller, and less branched than in pure F. heteroclitus.	Red yolk chromatophores much fewer also smaller and less branched than in other three forms.
2. Black yolk chromatophores large and of a general polygonal shape, processes usually wanting.	Black yolk chromatophores similar to those of previous class but a little smaller and with many minute processes.	Black yolk chromatophores similar to those of pure F. heteroclitus in early stages.	Black yolk chromatophores smaller, and with more and longer branches than in pure F. heteroclitus.
3. First crop of head chromatophores present.	First crop of head chromatophores present.	First crop of head chromatophores present.	First crop of head chromatophores absent.
4. Lateral line with a continuous line of red chromatophores at and before hatching.	Lateral line about the same as in previous class but the red chromatophores begin to fade sooner.	Lateral line about the same as in pure F. heteroclitus.	Lateral line without red chromatophores.
5. Lateral line with none or but few black chromatophores upon hatching.	Lateral line with a few more black chromatophores upon hatching than in pure F. heteroclitus.	Lateral line with comparatively few black chromatophores.	Lateral line with about 50 or 60 black chromatophores upon hatching.
6. When the yolk chromatophores first appear there are quite or nearly as many on the surface of the yolk sac opposite to the embryo as on any other part.	Yolk chromatophores at first about evenly distributed as in previous class.	Yolk chromatophores at first absent or decidedly fewer in that part of the yolk sac opposite to the embryo.	Yolk chromatophores first appear at the sides of the embryo, and are entirely absent from the hemisphere opposite to the embryo.
7. First yolk chromatophores appear earlier than in any of the other forms.	First yolk chromatophores appear later than in previous but earlier than in subsequent forms.	First yolk chromatophores appear later than in previous, but earlier than in following form.	First yolk chromatophores appear later than in any of the other forms.

number though still remaining in the vicinity of the embryo. After a few days their number again decreased markedly, apparently by incorporation within the embryo, so that they could only be found in a small proportion of the eggs, and in these not more than two or three cells to each yolk sac. There is no doubt then, that, so far as the *number* of these cells is concerned, there is a well marked Mendelian dominance, but as it is almost impossible to count them accurately I cannot be sure that in the hybrids the number of red yolk chromatophores is not slightly less than in pure *F. heteroclitus*.

An examination of the red yolk chromatophores in the four forms for *size* showed that the chromatophores of *F. majalis* were usually much smaller than those of any of the other forms; but that these cells in the *F. majalis* egg hybrid were slightly smaller and in the *F. heteroclitus* egg hybrid considerably smaller than in the pure *F. heteroclitus* (compare figs. 13 and 14; 15, 16, and 17; 18 and 19; 20 and 21; 22 and 23). As in most cases where the hybrids showed a condition intermediate between those of the two pure species it was the *F. heteroclitus* egg hybrid which was most like the pure *F. heteroclitus*; this case in which the *F. majalis* egg hybrids were most like the pure *F. heteroclitus* deserves special emphasis.

In *shape* the red yolk chromatophores of both hybrids approximated closely to the dominant condition of the pure *F. heteroclitus*. When they first appeared, these chromatophores in all four forms had about the shape of the red yolk chromatophore in fig. 13. In the pure *F. majalis* the red yolk chromatophores even at the period of their maximum complexity (fig. 17) did not progress far beyond this early shape; while the chromatophores of the three other became much larger and more complex. The *F. heteroclitus* egg hybrids usually had red yolk chromatophores of exactly the same shape as the pure *F. heteroclitus* (compare figs. 18 and 19, 20 and 21, 22 and 23). The *F. majalis* egg hybrids also in many cases had cells of exactly the same shape and almost the same size, as those of the pure *F. heteroclitus*. In some cases, however, the processes of these cells in the hybrids were fewer and the central body less extensive (fig. 9).

## 2. *Black yolk chromatophores*

For the first few days of their existence the black yolk chromatophores of both hybrids were identical with those of the pure *F. heteroclitus* in shape and the differences in size could apparently be entirely accounted for by differences in the ages of the cells. Compare figs. 13 and 14, 18 and 19 for the resemblance between the pure *F. heteroclitus* and the *F. heteroclitus* egg hybrid and figs. 8 and 11 for the resemblance between the pure *F. heteroclitus* and the *F. majalis* egg hybrid. In the pure *F. majalis* these cells were on the whole smaller and with more and longer processes than in the other three forms (see figs. 10, 12). This difference in *F. majalis* was most pronounced in those cells which were nearest to the embryo, as in fig. 10; the cells that were farther from the embryo (fig. 12) differed less in shape and size from those of *F. heteroclitus*.

We have seen that for the early stages there was complete dominance of the *F. heteroclitus* condition, as has been stated by Newman.<sup>4</sup> With further development, however, characteristic differences appeared so that each form could be distinguished by the appearance of the black yolk chromatophores alone.

In the pure *F. heteroclitus* these chromatophores soon crept onto the blood vessels, forming a coarse reticulum in which the clear spaces between the chromatophores were in general equal to or wider than the space occupied by the chromatophores themselves. There were very few branches traversing these clear spaces.

In the *F. heteroclitus* egg hybrid the chromatophores did not hug the vessels so closely so that the width of the colored meshes was usually about twice the thickness of the clear spaces between them. These clear spaces were also usually thickly traversed by a feltwork of fine processes (fig. 23). The color of this reticulum was also paler than in the pure *F. heteroclitus*, apparently because the chromatophores were spread over a greater area.

In the *F. majalis* egg hybrid there is, on account of the greater size of the egg, a greater space to be covered by the chromatophores. Together with this factor there was a much less pro-

<sup>4</sup> Jour. Exp. Zool., vol. 5, p. 550.

nounced tropism of the chromatophores for the blood vessels, so that here no characteristic chromatophore reticulum was formed. In addition there was much variation in the shape of the chromatophores during these later stages; some of them still retained their previous characteristic polygonal shape, while others developed long processes like the red yolk chromatophores or the black yolk chromatophores in the pure *F. majalis*.

In pure *F. majalis* the black yolk chromatophores were characterized by their long and numerous processes also during these later stages.

In considering these later deviations from the complete dominance of the early stages it might be thought that we had here two intermediate conditions manifesting themselves in the hybrids due to a delayed influence of some 'branching factor' derived from *F. majalis*. For in both cases the deviations are in the direction of the more profuse branching characteristic of *F. majalis*. I think, however, that in this case the deviations from complete dominance are due mainly to a diminution of the tropism of the chromatophores for the blood vessels discovered by Loeb.<sup>5</sup> This reduced chemotropism is probably due to a change in the contents of the blood vessels resulting from the diminished yolk assimilation in the hybrids which will be discussed later. However, no matter what its cause, there can be no doubt that in both the hybrids the yolk chromatophores are less closely approximated to the blood vessels than in either of the pure forms. Now when in

<sup>5</sup> Jour. Morph. 1893, viii, 161; Arch. f. d. ges. Physiol. liv, 525. Loeb at first thought that this tropism was chemotropism due principally to oxygen. Later he was inclined to consider that it might be stereotropism. Among the embryos in this series it was noticed in a number of cases that after the circulation had become well established and the chromatophore network was well formed that the blood accumulated in some one region and stopped circulating through the vessels, although the heart still continued beating normally for several days. When this stoppage of the circulation took place in a comparatively early stage it was seen on several occasions that the chromatophores left the vessels and became again uniformly distributed over the surface of the yolk. As the vessels were still present it must be concluded that chemotropism and not stereotropism is responsible for the creeping of the chromatophores on to the blood vessels. When the circulation stopped in later embryonal life the chromatophores usually continued to remain on the blood vessels even several days after the blood had ceased to circulate.

pure *F. heteroclitus* this chemotropism of the chromatophores for the blood vessels is destroyed by the stoppage of the circulation the black chromatophores of this form also develop more branches than usual and may even in extreme cases (such as fig. 24) simulate closely the chromatophores of the pure *F. majalis*. This instance shows very clearly the importance of an analysis of the mechanism of heredity insisted upon by Loeb in 1898<sup>6</sup> and more recently by Newman.

### 3. *Head chromatophores*

One of the factors which influenced Newman in arriving at the conclusion that in the hybrids between *majalis* and *heteroclitus*, blended inheritance predominates is the manner of the appearance of the head pigmentation. Newman found, and I have confirmed his results, that the head pigment appears first on the pure *F. heteroclitus*; next on the *F. heteroclitus* egg hybrid; next on the *F. majalis* egg hybrid; and last on the pure *F. majalis*. Now, while this is true, still a closer analysis of this process has furnished what is probably the clearest case of Mendelian dominance encountered in this whole study.

In the pure *F. heteroclitus*, and in both hybrids the black head chromatophores appeared in two crops separated by an interval of about two days. In the pure *F. majalis*, on the other hand, this first crop was wanting and the second crop appeared at about the same time that it did in the pure *F. heteroclitus*.

The first head chromatophores appeared on the first or second day after the first appearance of the yolk chromatophores. They were first seen on the sides of the brain, having probably migrated in from the yolk, and wandered onto the dorsum of hind and mid-brain, only in rare cases reaching the fore-brain. Various stages in the development of these cells are shown in figs. 1 to 6. As soon as they reached the dorsum of the brain they began to expand and finally became very conspicuous objects (fig. 5). Figures 2 and 3 which show these chromatophores before and after an interval of three and a half hours give some idea of the rapidity with which this migration and expansion takes place.

<sup>6</sup> Marine Biol. Lab. Lectures for 1897 and 1898, pp. 227-229.

In both hybrids these head chromatophores appear in essentially the same way that they do in the pure *F. heteroclitus*; there are, however, minor differences. In the hybrids they appear a little later, and they remain for a day or more on the sides of the brain without migrating onto its dorsum instead of only for several hours as in the case of the pure *F. heteroclitus*. Even after the chromatophores of the hybrids have reached the dorsum of the brain they do not look like any stage in the development of the same cells in the pure *F. heteroclitus*, for they are smaller and there are more of them. The comparative numbers of these cells for ten fish each of the pure *F. heteroclitus* and the *F. heteroclitus* egg hybrid are given in table 2. The differences while not great are, I think, significant. In the *F. majalis* egg hybrid the size and numbers of these cells are essentially similar.

TABLE 2

NUMBERS OF HEAD CHROMATOPHORES													AVERAGE
Pure <i>F. heteroclitus</i>	{ on mid-brain...	2	0	0	1	5	1	2	0	0	4		1.5
	{ on hind-brain...	5	4	3	4	4	4	3	2	0	5		3.4
<i>F. heteroclitus</i> egg hybrid.....	{ on mid-brain...	6	6	5	1	3	0	6	5	10	4		4.6
	{ on hind-brain...	5	4	2	3	4	7	4	6	3	4		4.2

In the pure *F. majalis*, on the other hand, there was no sign of this first crop of head chromatophores except in one case, out of several hundred embryos examined; and in this case the first crop was represented only by a single cell. With this single exception the first chromatophores to appear on the head of *F. majalis* were similar in all respects to the second crop of the other forms.

The second crop of head chromatophores in the pure *F. heteroclitus* and the two hybrids always appeared at a distinct interval after the first crop. This interval varied from one to four days. In the hybrids this crop usually appeared one or more days later than in the pure *F. heteroclitus*; but in the pure *F. majalis* it appeared at the same time as in the other pure form. In all four forms the method of development of this crop was the same, and was different from that of the first crop. As far as could be seen



these cells developed *in situ*; appearing first as faint, grey, thin and well branched cells scattered all over the dorsum of the brain. Soon after their first appearance their color became much deeper and they began to expand rapidly, so that after several days it was no longer possible to distinguish them from the chromatophores of the first crop. Fig. 6 shows the appearance of this second crop of head chromatophores in the same embryo which is represented nineteen hours earlier in fig. 5. A little later stage after the chromatophores of the second crop have become so large that they cannot always be certainly distinguished from those of the first crop is shown for the *F. majalis* egg hybrid of series 7 in fig. 9. To be compared with this last figure is fig. 10 taken from an embryo of the pure *F. majalis* also of series 7 and having the same age as the embryo figured in fig. 9. The entire absence of anything at all corresponding to the first crop of chromatophores is very evident.

It is very clear then that the *F. heteroclitus* character 'presence of the first crop of head chromatophores' dominates over the *F. majalis* character 'absence of this crop.' Furthermore, since the second crop of chromatophores appears in *F. majalis* synchronously with its appearance in *F. heteroclitus*, the delayed appearance of both crops in the hybrids cannot be considered a condition intermediate between that of the two pure forms.

#### 4. Red chromatophores of the lateral line

In the pure *F. heteroclitus* and the *F. heteroclitus* egg hybrids at the time of hatching and the *F. majalis* egg hybrids (which usually do not hatch) at about the same time the lateral line is mapped out by a series of about twenty conspicuous red chromatophores the characteristics of which are shown in figure 27. In all of these three forms these cells were very similar in appearance, and there can be no doubt that they are essentially the same kind of cells as the red yolk chromatophores. In the pure *F. majalis* these red chromatophores were not present on the lateral line at hatching time. There was, however, a series of very

indistinct pale cells showing white with a closed diaphragm which for a time I took to represent the red chromatophores. Later, however, it was found that these cells were probably the processes of the black chromatophores from which the pigment had withdrawn itself. For, all over fishes that were slightly older similar cells were found, with beautiful branched processes showing white by reflected light, and at the center of almost every one a small mass of contracted black pigment. We have then at the time of hatching what appears to be a clear case of the dominance of the *F. heteroclitus* character 'presence of red chromatophores' over the *F. majalis* character 'absence of red chromatophores.'

Immediately after hatching, however, this state of affairs began to change, for the red chromatophores began to fade, and when the fish were fed well had entirely disappeared in three or four days. When the fish were starved these chromatophores were visible in some cases for several days longer. These pigment cells did not contract or die but usually remained well branched and expanded as long as they were visible. The pigment, however, faded until it could only be seen in a few of the cell processes, usually lasting longest at the tips of these processes. Then it became practically invisible by transmitted, though still visible with reflected light; and finally could not be made out at all.

The possibility that in order to be visible this pigment needs something that it had been obtaining from the yolk but which is absent in the ordinary food naturally suggested itself, and perhaps receives some support from the fact that the pigment faded sooner when the fish was fed. But on the other hand, this rapid fading might equally well have been due to a general acceleration of development due to the feeding. An attempt was made to test the matter by feeding yolk but the close of the breeding season prevented conclusive results.

### 5. *Black chromatophores of the lateral line*

The pure *F. majalis* upon hatching and shortly before hatching had a series of from 40 to 60 black chromatophores along the lateral line (fig. 25). There were usually two chromatophores to the segment. One of these was near the surface of the fish and expanded in a plane parallel to the surface. The other was situated farther from the surface upon the septum in the frontal plane separating the dorsal from the ventral musculature. This second chromatophore expanded in the plane of this septum.

The pure *F. heteroclitus* at hatching time and before, usually had no black chromatophores at all along the lateral line. Perhaps ten per cent had one or two black chromatophores along the lateral line, and a much smaller percentage had more. But none were seen which had more than ten or twelve black chromatophores in this place. Upon the first day after hatching, however, 80 per cent or 90 per cent of the fish were found to have black chromatophores varying in number from 1 to 26 and averaging about 8. During the next few days this increase continued until all the fish had from 20 to 30 black chromatophores along the lateral line.

The *F. heteroclitus* egg hybrids were on the whole similar to the pure *F. heteroclitus*, but exhibit a slightly intermediate condition as the black chromatophores begin to appear on the lateral line a little earlier than in the pure *F. heteroclitus*. Thus at hatching time the hybrids usually had enough chromatophores so that above the anus, when expanded, they made a continuous line of black, with scattered black cells posterior to this region. Fig. 26 represents part of a fish of this kind, in which, however, the chloretone given to quiet the animal has caused the chromatophores to contract slightly and thus break up the continuous black line which was originally present. In the same series a comparison of the lateral lines a few days after shows that at this time also the hybrids maintained their lead in the development of the black chromatophores along the lateral line. At that time ten of the hybrids averaged 29.2 black chromatophores to a

lateral line with the extremes at 21 and 34; while ten of the pure form averaged 18.6 with extremes at 3 and 29. In this series the *F. heteroclitus* egg hybrids hatched at the same time as the pure *F. heteroclitus* so that these comparisons were made at the same age as well as the same stage and the difference in favor of the hybrids cannot be due to their greater age.

In the *F. majalis* egg hybrids the black chromatophores on the lateral line behaved quite similarly to those of the *F. heteroclitus* egg hybrid. But as these hybrids usually never do hatch a precise determination of the hatching time could not be made.

We have then so far as this character is concerned a well marked difference between the two species at the time of hatching and an incomplete dominance of the *F. heteroclitus* condition in the hybrids; but since the stages before and after hatching have not been sufficiently studied it cannot be told whether both species go through exactly the same series of changes, merely differing in their rate, or to what extent the hybrids are intermediate between the two pure forms.

#### 6. *Distribution of yolk chromatophores*

At the time of their first appearance both kinds of yolk chromatophores in the pure *F. heteroclitus* and the *F. heteroclitus* egg hybrids were found to be distributed over the whole surface of the yolk, and the region opposite to the embryo had quite as many or nearly as many chromatophores as any other region. In the pure *F. majalis*, on the other hand, almost the whole of the yolk hemisphere opposite to the embryo was free from chromatophores; and it took a number of days before the migration of pigment cells into this region became noticeable. The *F. majalis* egg hybrids presented an intermediate condition, for most of them had a small chromatophore free area in the region opposite to the embryo, and the others had fewer chromatophores than usual in this region. Figs. 11 and 12 show something of the differences between these last two forms. Later on the migration of chromatophores filled these empty areas with pigment cells and obliterated the difference between the various forms.

*7. Rate of development of yolk chromatophores*

In the pure *F. heteroclitus* the black yolk chromatophores first appeared when the heart was beginning to beat and before a circulation had been established; also before the fore-brain had acquired any lumen. The embryo had about twelve somites. The red chromatophores could usually not be seen until the next day.

In the *F. heteroclitus* egg hybrids the black yolk chromatophores also appeared at the time when the heart was first beating and before the circulation had started. In this form, however, the heart-beat and circulation started a little later than in the pure *F. heteroclitus*. At this time the fore-brain of the embryo usually had something of a lumen (condition was intermediate between figs. 1 and 2). Accordingly in this form the first appearance of the yolk chromatophores was later in time and also at a later stage in the development of the embryo.

In the *F. majalis* egg hybrids the yolk chromatophores did not appear until after the circulation was established, and until the embryo had a large lumen in the fore-brain like fig. 4.

In the pure *F. majalis* the yolk chromatophores appeared twelve to twenty-four hours later than in the *F. majalis* egg hybrids, at a time when the embryo was in a stage about half way between those represented in figs. 4 and 5.

Thus it is seen that both with respect to the time, and with respect to the development of the embryo, the hybrids had rates of development intermediate between those of their parent forms, and there was no indication of Mendelian dominance. The discovery, however, of factors which necessitated a Mendelian interpretation of the development of the head pigmentation, which, at first sight appeared exactly similar to this case of yolk pigmentation, makes one suspect that more study may result also in a Mendelian interpretation of the rate of development of the yolk chromatophores.

Although in this case the intermediate position of the hybrids and the lack of dominance is most evident, the same phenomenon is seen to a less degree in the development of the black chroma-

trophores of the lateral line, and the arrangement of the black yolk chromatophores, where incomplete dominance is associated with differences in rate of development. Newman's results also point in the same direction, though he does not mention this contrast between the absence of dominance in characters connected with the rate of development and the presence of dominance in other characters. Thus most of the characters which Newman investigated were concerned with the rate of development and in most of them he found 'blended heredity.' Thus we see that in general characters connected with the rate of development show blended heredity, and it may be that such characters are so intimately connected with extra nuclear substances such as the yolk that complete dominance is not obtainable.

#### LATER DEVELOPMENT

This later development concerns only the two parent species and the *F. heteroclitus* egg hybrid for the *F. majalis* egg hybrid was never found to live longer than two days after hatching. Newman found that none of this form hatched; but in these experiments, usually a few fish hatched in each series, perhaps a dozen in all. In all of these the hatching seemed premature, the yolk sac had not been absorbed, and the fish died a little later.

In the other forms the chromatophores began to contract shortly after hatching, probably because they then came under the influence of the nervous system. As the amount of contraction varied with the environment, and with the condition of the fish, close comparison of color patterns, and of size and shape of the chromatophores was no longer possible. The motions of the fish, for it was usually not safe to risk narcotization, also made exact comparisons difficult, I think, however, that it may be safely said that the only changes that have taken place since hatching are all in the direction of making the three forms more like each other, until at the present time, three months after hatching I can find no characters which will distinguish any one form from the other two. They all have developed much more pigment of the black and greenish yellow kinds, especially in the dorsal region;

and they all have developed from three to six or seven transverse black bands due both to an increased number of chromatophores and an increased expansion of the chromatophores in the region of the bands. Between the bands there are at present usually not more than one black chromatophore to the scale, while in the more pronounced bands there may be as many as four or five chromatophores to the scale. The present appearance of the fish is much like that of the females of *F. heteroclitus* shortly after the breeding season, when indistinct transverse dark bands may be seen.

#### CHARACTERS OTHER THAN THOSE OF PIGMENTATION

As regards the rate of the development of the embryo my observations confirm those of Newman on most points. The development of the *F. heteroclitus* egg hybrid was slower than that of its maternal parent; and the development of the *F. majalis* egg hybrid, during the early stages was faster than that of the pure *F. majalis*. After hatching the *F. heteroclitus* egg hybrid seemed more vigorous and grew faster under like conditions than either of the pure forms.

The failure of the *F. majalis* egg hybrids to develop well during the later stages seemed to depend primarily upon the poor digestion of the yolk in this form. The first considerable difference that could be seen between the pure *F. majalis* and the *F. majalis* egg hybrid was that in the hybrid the yolk was not digested away from under the embryo as rapidly as in the pure form. A result of this appeared to be that, when the heart was forming, the vesicle underneath the embryo was very shallow and the normal anterior curvature of the head did not take place (compare figs. 28 and 29). These two factors seemed to be responsible for the fact that when the heart first began to beat in the hybrid it was closely pressed to the ventral side of the embryo (fig. 28) and did not extend across the vesicle making an angle of nearly 90° with the embryo as in the pure form (fig. 29). Consequently from the very first the heart in the hybrid was much stretched and on the next day when the circulation had become established the heart

in the hybrid was much longer, narrower, and less efficient than in the pure form. In the hybrid the heart never did get over this initial handicap but was stretched farther as development proceeded until finally shortly before the embryos died it had assumed the appearance shown in fig. 30, and at each beat was propelling only a very small amount of blood through the vessels. It seems then that a slight retardation in the digestion of the yolk led to such an increase in the distance between the points of attachment of the heart to yolk sac and embryo, that the heart could not grow fast enough to catch up, but remained permanently disabled.

#### SUMMARY

1. While it had been generally assumed that in hybrid embryos the inheritance was either maternal or paternal, Loeb, King and Moore have called attention to the fact that for the hybrid sea-urchin embryo we find dominance of individual characters as in the adult. For *Fundulus* hybrids Newman found a dominance of a few individual characters, but usually found the characters intermediate between those of the two parent species. In this study, which is concerned mainly with the pigment characters of *Fundulus heteroclitus*, *F. majalis*, and their hybrids, dominance of individual characters has been found in most cases, as in the following characters:

a. The character—presence of many large red yolk chromatophores (*F. heteroclitus* condition) is dominant over the character—presence of few small red yolk chromatophores (*F. majalis* condition).

b. The size and shape of the black yolk chromatophores of *F. heteroclitus* is dominant over the size and shape of these same cells characteristic of *F. majalis*.

c. The presence of a first crop of head chromatophores appearing before the majority of the head chromatophores (*F. heteroclitus* condition) is dominant over the absence of this crop of head chromatophores (*F. majalis* condition).

d. The presence of red chromatophores along the lateral line at hatching time, or shortly before it (*F. heteroclitus* condition)



is dominant over the absence of red chromatophores at the same time (*F. majalis* condition).

2. In all of the above characters which concern mainly the presence or absence and not the time relations of the pigment characters the dominance is very evident, though often to a certain extent incomplete.

3. In the following characters, however, which are mainly concerned with the time relations the dominance is much less complete, or wanting altogether.

a. At hatching time *F. majalis* has a row of 50 or 60 black chromatophores along the lateral line, while in *F. heteroclitus* there are usually no chromatophores on the lateral line until hatching time when they begin to appear and gradually increase in number. The hybrids are intermediate, having at hatching time about 15 or 20 black chromatophores on the lateral line, and developing additional cells more rapidly than in the pure *F. heteroclitus*.

b. When the yolk chromatophores in *F. heteroclitus* first appear they are evenly distributed over the whole yolk sac; while in *F. majalis* they are absent from the yolk hemisphere farthest from the embryo. The *F. heteroclitus* egg hybrid is like its maternal species, while the *F. majalis* egg hybrid is intermediate having, on the side of the yolk sac opposite to the embryo, a small area in which the chromatophores are either absent or fewer than elsewhere.

c. A more perfect case of blended inheritance exists, as Newman has already shown, for the time of appearance of the yolk chromatophores. In *F. heteroclitus* these cells appear much earlier (both with respect to time, and with respect to the stage of development of the embryo) than in *F. majalis*. In the hybrids the time of appearance of these cells is intermediate, but each hybrid resembles its maternal more than its paternal parent.

4. It appears then that the presence of certain pigment characters dominates over their absence or lesser development; while for the time relations of these pigment characters blended heredity holds. This difference may be fundamental or due to an incomplete analysis of the time relations.

5. A similar case of blended inheritance, described by Newman at the time of first appearance of head pigmentation, was found to be actually a case of the combination of two crops of head chromatophores. The second crop appears in both species and hybrids. The first crop is present in *F. heteroclitus* and both hybrids and hence its presence is a dominant in the Mendelian sense.
6. Immediately after hatching the characters which have served to distinguish the four forms begin to disappear so that after a few months both pure species and the hybrids look practically alike.

All camera drawings from living fish. The opaque red chromatophores are colored in red. Dotted lines indicate blood vessels. *H* = heart.



Fig. 1. Pure *F. heteroclitus*, four days old, showing first chromatophores.  $\times 25$ .

Fig. 2. Pure *F. heteroclitus*, three and one half days old, circulation started not more than a few minutes before drawing began.  $\times 25$ .

Fig. 3. Brain of same embryo drawn in fig. 2 but three and one half hours later.  $\times 25$ .



4



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6



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Fig. 4—Pure F. heteroclitus, five days old, showing the brain chromatophores and half of the yolk chromatophore ring about the embryo. *H*—heart.  $\times 25$ .

Fig. 5—Pure F. heteroclitus, six days old, showing the first crop of brain chromatophores well expanded. Red brain chromatophores not drawn.  $\times 25$ .

Fig. 6—Same embryo drawn in fig. 5, but nineteen hours later showing the first appearance of the second crop of brain chromatophores. The four cells of the first crop still recognizable.  $\times 25$ .

Fig. 7—Same embryo drawn in fig. 6, but forty-eight hours later. Shows the fusion of the head chromatophores.



B



9

Fig. 8. Pure *F. heteroclitus*, three and one-half days old. Heart beating but no circulation. Shows the yolk chromatophore ring about the edge of the hallow vesicle under the embryo.  $\times 25$ .

Fig. 9. *F. majalis* egg hybrid, seven days old. Shows both crops of head chromatophores.  $\times 25$ .



Fig. 10. Pure *E. majalis*, seven days old. (Belongs to same series as embryo of fig. 9.) Shows absence of the first crop of head chromatophores; also characteristic shape of black yolk chromatophores. ( $\times 25$ ).

Fig. 11. *E. majalis* egg hybrid, six days old. (Shows shape and arrangement of yolk chromatophores. ( $\times 49$ ).

Fig. 12. Pure *E. majalis*, six days old. (Shows the absence of yolk chromatophores on the part of the yolk away from the embryo. Many chromatophores near the embryo were not drawn as they were much obscured by the yolk globules. ( $\times 49$ ).

Fig. 13. Pure *E. heteroditus*, four days old. (There were no blood vessels near the cells figured. Shows characteristic early chromatophore group, before they have migrated onto the blood vessels. Embryo same stage as fig. 2. ( $\times 111$ ).

Fig. 14. *E. heteroditus* egg hybrid, four days old; has good circulation. (Embryo same stage as fig. 2. Shows characteristic group of yolk chromatophores on the side of the yolk sac, opposite to the embryo, where the blood vessels have just become established. ( $\times 111$ ).

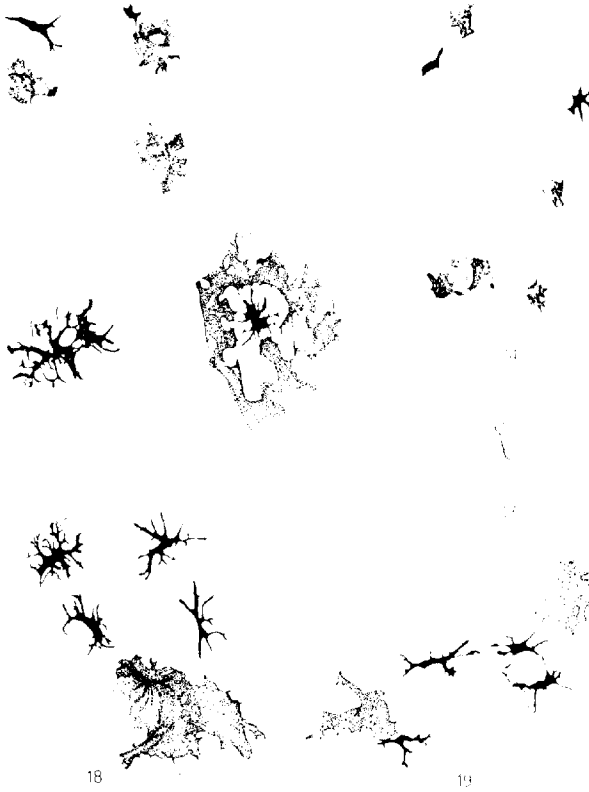


Fig. 15. Pure *F.* heteroclitus, twelve days old. Fully developed red chromatophore near the eye of embryo. Dotted lines indicate blood vessels.  $\times 75$ .

Fig. 16. *F.* heteroclitus egg hybrid, twelve days old. Fully developed red yolk chromatophore in center of clear space in black chromatophore reticulum.  $\times 75$ .

Fig. 17. Pure *F.* majalis, thirteen days old. Characteristic red yolk chromatophore on blood vessel.  $\times 75$ .

Fig. 18. Pure *F.* heteroclitus, six days old. Group of chromatophores beginning to form chromatophore reticulum on blood vessels near left eye.  $\times 114$ .

Fig. 19. *F.* heteroclitus egg hybrid, six days old. Same series and same age as embryo of fig. 18. Group of chromatophores on blood vessel near left eye.  $\times 114$ .

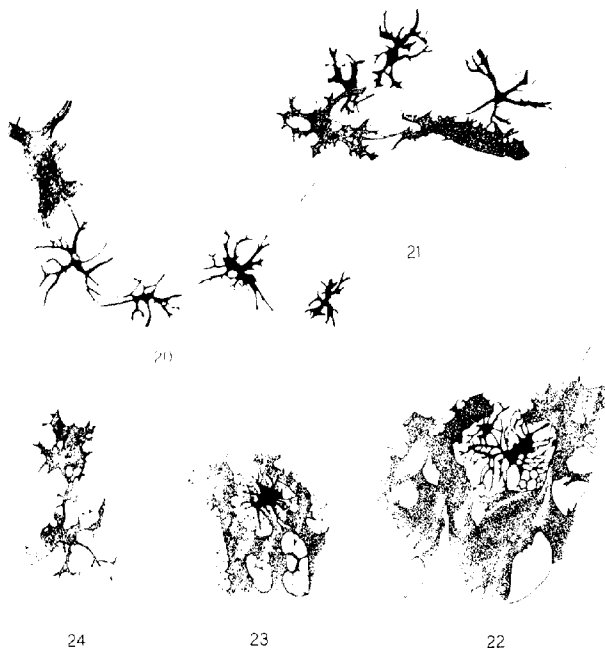


Fig. 20. Pure *F. heteroclitus*, seven days old. Same embryo as in fig. 18. Chromatophores on blood vessel near left eye. To show increase in size and complexity during last twenty-four hours.  $\times 114$ .

Fig. 21. *F. heteroclitus* egg hybrid. Same embryo as in fig. 19. Chromatophores on blood vessel near left eye. Note increase in size of red chromatophores and the beginning of the small processes of black chromatophores.  $\times 114$ .

Fig. 22. Pure *F. heteroclitus*, eleven days old. Typical fully developed red chromatophore among the black chromatophore reticulum.  $\times 114$ .

Fig. 23. *F. heteroclitus* egg hybrid, eleven days old. Of same age and series as embryo in fig. 22, with which it is to be compared. Note double layer of chromatophores and fine black reticular processes.  $\times 114$ .

Fig. 24. Pure *F. heteroclitus*. Most of the eggs of this lot had hatched, but in this embryo the circulation had stopped though the heart was still beating, and the blood vessels distinct and full of blood. The yolk chromatophores have begun to lose their arrangement on the blood vessels and have developed much longer branches than any normally seen in this species.  $\times 114$ .

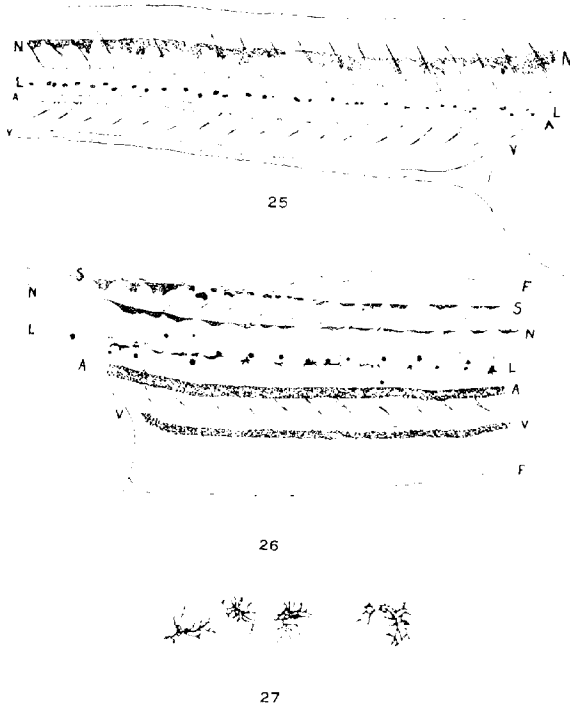


Fig. 25—Pure *F. majulis*, thirty-five days old, just hatched. Shows the line of chromatophores along the dorsal surface of the nerve cord, *N*, *N*, which were expanded; and the contracted chromatophores of the lateral line, *L*, *L*. *A*—*A*—Aorta, *V*—*V*—Ventral vein. Neither the chromatophores on these last two vessels nor on the dorsal surface of the fish have been drawn in.  $\times 25$ .

Fig. 26—*F. heteroclitus* egg hybrid, sixteen days old, just hatched. *S*—*S* line of chromatophores under dorsal skin, partly contracted by the narcotization. *A*—*A* chromatophores on nerve cord, partly contracted; *L*—*L* lateral line. All the red chromatophores contracted, black chromatophores partly contracted at left where they formed a complete line when the drawing was begun. In the center of the lateral line the chromatophores are expanded; and on the right some of them are completely contracted on account of another dose of narcotic. *A*—*A*—Aorta, *V*—*V*—Ventral vein. Chromatophores on these last two vessels drawn in from another uncontracted fish.  $\times 25$ .

Fig. 27—Pure *F. heteroclitus*, just hatched. Shows red opaque chromatophores of the lateral line.  $\times 127$ .





Fig. 28. *F. majalis* egg hybrid, four days old. Shows position of the heart which has just begun to beat, and the shallow vesicle, V, underneath the embryo.

Fig. 29. Pure *F. majalis*, four days old. Same series and age as embryo in fig. 28. To show the greater depth of the vesicle, V, under the embryo and the normal position of head and heart. Heart has just begun to contract.

Fig. 30. *F. majalis* egg hybrid, twenty-four days old. Dissected out of the egg shell at a time when a few fish of the same lot had hatched and when many had died. Shows vesicle V, under the embryo and the greatly stretched heart within it.

# LONGEVITY IN SATURNIID MOTHS: AN EXPERIMENTAL STUDY

PHIL RAU AND NELLIE RAU

FIVE CHARTS

## INTRODUCTION

The observations and experiments herein recorded upon the longevity of some of the Saturniid moths were undertaken in order to discover the value of some of the theories that have been advanced to account for the duration of life. Much of the theorizing is based upon the insufficient and inaccurate knowledge of the ages attained by different organisms and the relation of such length of life to their reproductive function, as well as to their environmental conditions. The attempt to place our knowledge of the duration of life upon a scientific basis demands the gathering of many data on many species, and on the mated and unmated individuals of both sexes.

With these needs in view we found some members of the family Saturniidae in sufficient numbers to suit our purpose. A more important reason for this choice was the fact that they have aborted mouth-parts, and the adult insects take no nourishment. This would eliminate the probabilities of curtailment of life due to insufficient or improper food.

The cocoons were gathered and carefully strung to trees where they could be subjected to the natural changes of the weather conditions during the winter. Just previous to the emerging time they were taken into a shed, the temperature of which varied but little from that of the outside. The imagines were kept under ordinary dome-shaped, wire dish-covers, which varied from 22 to 32 inches in circumference.

This work falls into the following divisions: The duration of life in:

1. *Samia cecropia*. 1910—178 insects from St. Louis cocoons.
2. *Samia cecropia*. 1911—112 insects from St. Louis cocoons. Cocoons placed in incubator.
3. *Samia cecropia*. 1911—42 insects from St. Louis cocoons. Imagines placed in ice-box.
4. *Samia cecropia*. 1911—283 insects from St. Louis cocoons.
5. *Samia cecropia*. 1911—133 insects from Long Island cocoons.
6. *Callosamia promethea*. 1911—170 insects from Creve Coeur Lake, Missouri, cocoons.
7. *Tropaea luna*. 1911—60 insects from St. Louis and Pike County, Missouri, cocoons.
8. *Telega polyphemus*. 1911—19 insects from St. Louis and Pike County cocoons.

#### REVIEW OF THE THEORIES

Before taking up the details of the work, it would be well to rehearse here briefly the various theories which have been advanced to account for the duration of life.

First among these is the theory of Weismann, that the duration of life of an organism

is really dependent upon adaptation to external conditions, that its length, whether longer or shorter, is governed by the needs of the species, and that it is determined by precisely the same mechanical process of regulation as that by which the structure and functions of an organism are adapted to its environment.<sup>1</sup>

I consider that death is not a primary necessity, but that it has been secondarily acquired as an adaptation. I believe that life is endowed with a fixed duration, not because it is contrary to its nature to be unlimited, but because the unlimited existence of individuals would be a luxury without any corresponding advantage. The above-mentioned hypothesis upon the origin and necessity of death leads me to believe that the organism did not finally cease to renew the worn-out cell material because the nature of the cells did not permit them to multiply

<sup>1</sup>Essays upon Heredity, 2 ed., vol. 1, p. 9. 1891.

indefinitely, but because the power of multiplying indefinitely was lost when it ceased to be of use.<sup>2</sup>

In answering the question (continues Weismann, *loc. cit.*, p. 20), as to the means by which the lengthening or shortening of life is brought about, our first appeal must be to the process of natural selection. Duration of life, like every other characteristic of an organism, is subject to individual fluctuations. . . . As soon as the long-lived individuals in a species obtain some advantage in the struggle for existence, they will probably become dominant and those with the shortest lives will be exterminated.

Lankester,<sup>3</sup> according to Romanes, has pointed out "a highly remarkable correlation between potential longevity in the individual and frequency of parturition." "This correlation he attributes to generative expenditure acting directly to the curtailment of life."

Romanes,<sup>4</sup> who like Weismann sees in the duration of life an adaptation, does not agree with Lankester that it is the generative expenditure "that causes the curtailment of life, but that it is the curtailment of life by Natural Selection, which causes the high generative expenditure within the lessened period."

In opposition to the utilitarian theory of Weismann is that of Götze,<sup>5</sup> that

. . . all animals are mortal, and reproduction is in itself the cause of death. Reproduction in Protozoa is preceded by encystation. In this condition the organism passes into a non-living condition, from which it revives with renewed youth and renewed life; a similar condition occurs in the egg of Metazoa, during a certain period in which it forms an unorganized, non-living body, composed of organic substances.

Eimer's<sup>6</sup> own opinion is that in the Metazoa as well as in the Protozoa the germ cells are immortal; only the soma dies.

"The latter is not really an end in itself, but rather its principal function is to ensure the maintenance of organic life, by favoring reproduction, by sheltering the germ-cells till their maturity, and in order to deposit them repeatedly; further, by the dispersal of the same in space,

<sup>2</sup> *Loc. cit.*, p. 25.

<sup>3</sup> *Comparative Longevity*, 1870, quoted by Romanes, *Monist*, vol. 5, p. 163, 1895.

<sup>4</sup> *Monist*, vol. 5, p. 163, 1895.

<sup>5</sup> Quoted by Eimer, *Organic Evolution*, p. 67, 1890.

<sup>6</sup> *Loc. cit.*, p. 68-69.

by incubation in the widest sense, and so on. Further it has the function of strengthening the power of endurance of the species by the inheritance of acquired characters." "Reproduction is unending growth. Not reproduction is the essential cause of death. . . . the soma is not really an end in itself, but rather its principal function is to ensure the maintenance of organic life by favoring reproduction.

Minot<sup>7</sup> seems to conclude that "the duration of life depends upon the rate of cytomorphosis. If that cytomorphosis is rapid the fatal condition is reached soon, if it is slow the fatal condition is postponed."

Flourens<sup>8</sup> thinks that the length of life of an animal is equivalent to five times its period of growth, while Nägeli<sup>9</sup> thinks that "natural death does not exist in nature, for trees more than a thousand years old perish not by natural death, that is to say natural decay of their vitality, but by some catastrophe."

Metchnikoff<sup>10</sup> says: "It is impossible to regard natural death, if indeed it exist, as the product of natural selection for the benefit of the species. In the press of the world natural death could hardly come into operation because maladies or the voracity of animals so frequently cause natural death."

While Morgan<sup>11</sup> says that "in some cases the length of life and the coming to maturity of the germ-cell may be, in some way, physiologically connected seems not improbable, but that this relation has been regulated by the competition of species with each other can scarcely be seriously maintained," he will "not pretend to say whether the mutation theory can or cannot be made to appear to give the semblance of an explanation of the length of life in each species."

Other theories of less importance are reviewed by Metchnikoff in "The Prolongation of Life" and "The Nature of Man."

<sup>7</sup> The Problem of Age, Growth and Death, p. 228, 1908.

<sup>8</sup> Quoted by Metchnikoff, Prolongation of Life, p. 40, 1908.

<sup>9</sup> Quoted by Metchnikoff, The Nature of Man, p. 265.

<sup>10</sup> Loc. cit., p. 267.

<sup>11</sup> Evolution and Adaptation, p. 371, 1908.

## OBSERVATIONS

In 1909 notes were made upon the duration of life in the *Cecropia* moth.<sup>12</sup> The observations were made upon only a small number however, so it was decided to carry the work on during 1910, on a much larger scale. New factors of much interest were discovered entering into the work of that year, so it was deemed best to continue an experimental investigation through a third year in order to get adequate and conclusive data on some of the phenomena appearing.

These three consecutive years of observations on the *Cecropia* were made upon material gathered from the same locality, River des Peres, St. Louis. The parallel work was carried on upon the *Cecropia* moths from Long Island, New York, in order to ascertain whether there were any unusual phenomena in the St. Louis material due to purely local conditions.

TABLE 1  
*Mean duration of life*

LOT	MATED ♂'s	UNMATED ♂'s	MATED ♀'s	UNMATED ♀'s	ALL MATED INSECTS	ALL UNMATED INSECTS	ALL ♂'s	ALL ♀'s	WHOLE POPULATION
<i>S. cecropia</i> . 1910.									
St. Louis (early)...	17.40	17.67	14.83	16.80	15.77	17.29	17.59	15.79	16.65
<i>S. cecropia</i> . 1910.									
St. Louis (late)....	10.62	10.52	9.46	10.50	9.90	10.51	10.56	9.76	10.14
<i>S. cecropia</i> . 1911.									
St. Louis.....	7.52	7.73	6.96	7.90	7.20	7.81	7.70	7.71	7.71
<i>S. cecropia</i> . 1911.									
New York.....	7.90	8.54	6.62	8.25	7.25	8.42	8.37	7.65	8.06
<i>S. cecropia</i> . 1911.									
Incubator.....	8.10	8.31	7.03	8.80	7.40	8.48	8.24	7.73	8.00
<i>S. cecropia</i> . 1911.									
Ice Box.....							17.58	19.39	18.60
<i>C. promethea</i> . 1911.	3.74	4.21	5.18	6.54	4.51	4.91	4.13	6.21	4.82
<i>T. luna</i> . 1911.....	4.60	6.07	6.60	5.80	5.60	5.96	5.86	5.96	5.90
<i>T. polyphemus</i> . 1911.							5.33	9.29	6.79

Table 1 brings together the means of all the lots of material, and will be referred to frequently for comparison.

<sup>12</sup> Trans. Acad. Sci. St. Louis, vol. 19, pp. 21-48, 1910.

The St. Louis *Cecropias* will be discussed in detail, and the other groups will be taken up later only for comparative evidence.

The mean duration of life of all the St. Louis *Cecropias* (under normal conditions only) for the three years was 10.61, 13.73 and 7.71 days. We are at once struck with the great variation, for in so brief a life a day is as a decade in the life of man. If now we can detect the reasons for these variations from year to year in the life of the population, it may lead us toward the discovery of the factors controlling the duration of life of the species.

In 1910 notes were based upon 178 insects from the 205 which emerged, (101 males and 104 females). Hardly was the work begun when a marked difference in the date of emergence was observed. The insects of that year began to emerge on April 13, a month earlier than in the year before or after.

Table 2 shows at a glance the marked correlation between the date of emergence and the duration of life of the animals; those which emerged early lived distinctly longer lives than those which appeared late in the season. The duration of life of the entire population varies from 5 to 25 days. The table clearly shows how all of the early emerging insects segregate to the long-lived lot, while those emerging late in the season fall under short lives. In fact, May 14 seems to be a distinct dividing line between the early emerging or long-lived groups, and the late or short-lived group. The late lot, taken separately, has practically the same dates of emergence and duration of life as the 1909 population.

It certainly seems remarkable that the population should split up in this fashion; the problem is most perplexing. The questions at once arise in our minds: Is long or short life hereditary? Is it regulated by climatic conditions? Are these results due to local conditions, and would the same be seen in material from other localities and in other species of the same family? Has each organism an "allotment" of a certain number of days, i.e., from the time of the fertilization of the egg to the death of the adult, and is a longer or shorter period in one of the early stages correlated with a shorter or longer life in the imago?

A cause for this early emergence is very uncertain to determine without tracing the duration of the different stages of the whole life cycle of each insect. But the fact that this abnormally

DURATION OF LIFE IN DAYS

DURATION OF LIFE IN DAYS	
1	1
2	1
3	1
4	1
5	1
6	1
7	1
8	1
9	1
10	1
11	1
12	1
13	1
14	1
15	1
16	1
17	1
18	1
19	1
20	1
21	1
22	1
23	1
24	1
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80	1
81	1
82	1
83	1
84	1
85	1
86	1
87	1
88	1
89	1
90	1
91	1
92	1
93	1
94	1
95	1
96	1
97	1
98	1
99	1
100	1



early emergence followed the exceptionally warm month of March at once leads one to suspect that the high temperature at that stage of their pupal development may have accelerated growth. The mean temperature for March, 1910, was  $57.5^{\circ}\text{F.}$ , as contrasted with  $44^{\circ}$  and  $47^{\circ}$  for the other two years, and the maximum reached was  $87^{\circ}$ .<sup>13</sup>

It was noticed that during some of the cold days of April and May the animals showed signs of extreme sluggishness, and while *Cecropias* in confinement seem to be inactive only during the day, those which were on hand when the cold snap came were extremely sluggish during both day and night. It was thought that the cold had a direct effect upon the duration of life, for when the animals were inactive, little or no reserve nutriment was consumed and this saving of vital energy, which is never replenished, may have prolonged their lives. A correlation clearly exists between temperature and longevity. We find that almost all of the long-lived insects (table 2) emerged between April 13 and May 10. These died at intervals between April 27 and May 29. The average of the daily mean temperatures for this period of 47 days was  $57.5^{\circ}\text{F.}$  The short-lived ones emerged between May 11 and June 17, and died between May 21 and June 28; the average of the daily mean temperature for this period of 49 days was  $68.1^{\circ}\text{F.}$  Therefore the average of the mean temperature was lower by  $11^{\circ}$  during the time when the long-lived insects existed.

Now when we tabulate these two groups separately (tables 3 and 4), we find the mean duration of life of the early lot to be 16.65 days, while that of the late ones which ran into warm weather, is only 10.14 days.

<sup>13</sup> To quote from the Monthly Meteorological Summary of the United States Weather Bureau: "The weather for March was very unusual. The mean temperature was  $57.5^{\circ}$  which is  $3.2^{\circ}$  higher than for any previous March and the temperature was continuously above the normal except on the 9th, 10th, 14th and 15th. The maximum temperature for the month was  $87^{\circ}$  and this has been exceeded but once in March in the history of the station. Freezing temperatures occurred on three days only. . . . The number of clear days, 22, is the highest ever observed in March, and the number of cloudy days, 2, the lowest. The sunshine was 79 per cent of the possible amount and was greatest for March since the records began.

All temperatures given in this paper are quoted from these reports.

The desire to test further the influence of temperature upon length of life led us to (1) the Incubator Experiments, and (2) the Ice-Box Experiments.

TABLE 3  
*Early emerging, long-lived Cecropias, 1910*

CLASS	DAYS																								T. MEAN								
	9	9.5	10	10.5	11	11.5	12	12.5	13	13.5	14	14.5	15	15.5	16	16.5	17	17.5	18	18.5	19	19.5	20	20.5		21	21.5	22	22.5	23	23.5	24	
Mated ♂'s.....	1				1	1							2				2		1	2	2								1	1	15	17.40	
Unmated ♂'s.....		1			2	1		1	1				3	3	1		1	1	3	2	1	3	2	2	1	1				1	132	17.67	
Mated ♀'s.....		1	1	1	2		1	1	3	3		1	2	1	2				1	1	3		1	1							36	14.83	
Unmated ♀'s.....	1		1		1		1	2	1	2	1			2	3	2			1	2											125	16.80	
All mated insects.....	1	1	1	1	3	1	1	1	3	3		3	2	1	2			3	1	4	2	3	1							10	11	41	15.77
All unmated insects.....	1		1	1	3	1	1	3	2	2	1	3	3	3	4	3	3	3	3	3	2	2	1	1	2	2					257	17.29	
All ♂'s.....	1	1	1		1	3	1	1	1	2	3	3	1	1	3	3	1	4	3	3	2	2	1	2	2	2				2	147	17.59	
All ♀'s.....	1	1	1	2	2	1	1	2	5	4	2	2	1	4	3	3	1	3	1	1									2	151	15.79		
Whole population.....	2	1	2	2	3	4	2	2	6	5	2	4	5	4	5	4	6	4	4	5	6	4	3	2	1	2	2	3	1	298	16.65		

TABLE 4  
*Late emerging, short-lived Cecropias, 1910*

CLASS	DAYS																	T. MEAN
	5	5.5	6	6.5	7	7.5	8	8.5	9	9.5	10	10.5	11	11.5	12	12.5	13	
Mated ♂'s.....	1		1		1		2	2	2	1					4		3	18 10.62
Unmated ♂'s.....			1		2	3	1	2	2	3				3	2	3		20 10.52
Mated ♀'s.....	1	3	1	2	2	4	3	3	1	2	1			2	2	1	1	1 30 9.46
Unmated ♀'s.....			1		3	2		1		1				2				1 12 10.50
All mated insects.....	2	3	2	2	1	2	4	5	5	3	3	1		6	2	4	1	1 48 9.90
All unmated insects.....			2		5	5	1	3	3	1	3	4	3		1			1 32 10.51
All ♂'s.....	1		1	1	2	3	3	2	4	1	3			7	2	6		1 38 10.56
All ♀'s.....	1	3	1	3	5	6	3	3	2	2	1	1	2	4	1		1	2 42 9.76
Whole population.....	2	3	2	4	1	7	9	6	5	6	3	4	1	9	6	7	1	2 80 10.14

#### INCUBATOR EXPERIMENTS, CECROPIA 1911

After the work of 1910 had given a clue that the climatic conditions may have been a factor in regulating the duration of life, it was thought that if by some method the cocoons could be controlled so that the imagines could be gotten in January or February and subjected to the climatic conditions of that season, some interesting results would be obtained.

Some 300 cocoons were placed in an incubator on September 20, the temperature of which was regulated to about 70° F.

(about the mean temperature for the period in 1910 when the moths emerged). Up to the 10th of January, nothing had emerged. At this time a living-room was obtained with a fairly even temperature. Up to the middle of February no results were obtained. It then became necessary to remove the entire lot to the basement, the temperature of which, while not recorded, was moderately uniform and distinctly higher than that out-of-doors. They were sprinkled with water at intervals of a few days. These details would not be worth recording but for the fact that it was expected that under these conditions the insects would probably emerge somewhat earlier than the normal time. On the contrary however, the 162 insects which emerged (72 males, 90 females), left the cocoon between June 5 and July 9, the latest period yet met with in *Cecropia* work.

This lot was gathered early and placed in the incubator very soon after pupation, while probably some of the cocoons still contained larvae. It seems that the constant even temperature conditions at this stage made the animals lethargic and indifferent to the normal development, when only a year before an unusually warm March had probably caused many to emerge sooner.

The warm March of 1910 caused an earlier emergence, but warmth was furnished to the insects at a period of their development when they were susceptible to its accelerating influences, but when it was given them at an early stage of their pupal development, counter results were obtained.

Can it be that they spun their cocoons in preparation for the cold of winter, but just about the time or even before they had left the larval stage their summer (i.e., high temperature) was resumed so they lacked the stimulus (cold) to start them promptly in their pupal development?

If we had reason to believe that the first animals to pupate are the last to emerge, this would easily explain in part the lateness of these in emerging, for they were all gathered very early in the pupating season, while many caterpillars were yet to be seen. We cannot believe, however, that this can be a full explanation for the phenomenon, for the lateness of these too far exceeds that of any others observed from the same region, either in captivity or free.

Another interesting feature was the degree of pigmentation displayed by these insects. While no measurement was made of the color or its distribution, the general darkness of this lot was clearly evident.

Out of the 162 insects which emerged from the incubator lot, records were kept on the duration of life of 112. The mean length of life for this lot compares well (table 1) with the results from the New York and St. Louis 1911 material. The following table gives the details of the duration of life of this lot.

TABLE 5  
*Incubator Cecropias*

CLASS	DAYS														T.	MEAN
	4	5	6	7	8	9	10	11	12	13	14	15				
Mated ♂'s.....		2	3	4	1	6		4							20	8.10
Unmated ♂'s.....		3	4	4	11	8	4	3	2						39	8.31
Mated ♀'s.....	1	3	10	9	2	8									33	7.03
Unmated ♀'s.....		1	1	4	3	5	2	1	2	1					20	8.80
All mated insects.....	1	5	13	13	3	14		4							53	7.10
All unmated insects.....		4	5	8	14	13	6	4	4	1					59	8.48
All ♂'s.....		5	7	8	12	14	4	7	2						59	8.24
All ♀'s.....	1	4	11	13	5	13	2	1	2	1					53	7.73
Whole population.....	1	9	18	21	17	27	6	8	4	1					112	8.00

#### ICE-BOX MATERIAL, CECROPIA 1911

Finding it impossible to get the incubator material to emerge during the winter, and wishing to ascertain definitely whether the duration of life is influenced by low temperature, a number of cages containing two insects each were placed in an ordinary household ice-box, the temperature of which varied from 9° to 12° C., and on a few occasions 15°. Forty-two insects were used in this experiment, and the duration of life varied from 6 to 32 days. Had the temperature been uniform in all parts of the ice-box, the 18 insects which lived 13 days or less would probably have lived longer. As it was, most of those which were kept on the top shelf nearest the ice compartment lived the longest. Correlation table 7 shows that their contemporaries lived the normal number of days.

Lack of facilities and material made it impossible to make more extended observations. This number, however, gives suf-

ficient evidence that long life in this case is a matter dependent upon climatic conditions.

TABLE 6  
*Ice-box Cecropias*

CLASS	DAYS																																T.	MEAN
	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32							
Males.....	1			2	2	1	2	3				1	2	1	1			1	1	2			1		1	1	1	24	17.58					
Females.....				1	2	1	2	3						1	1	3		1	1	1	1			3				18	19.39					
All.....	1			3	2	3	3	6			1	2	1	2		1	1	3	1	1	1		4	1	1	4	42	18.60						

This ice-box material compares well in the duration of life with the early lot of 1910, but the fact must not be forgotten that in spite of being kept in the ice-box at a temperature of 9 to 11° C., the insects were not so sluggish as they were during some of the much colder days of 1910. Could the refrigerator have been properly regulated, no doubt a greater period of life could have been attained. That the animals were far from inactive was evident from the worn condition of the wings. Copulation and oviposition also occurred while under these conditions. The activities of these may not have been normal; still the profound sluggishness which was observed during the cold spells of the year before did not occur.

#### ST. LOUIS MATERIAL, CECROPIA 1911

The object of this work was to see if the population would split up into long- and short-lived groups as it did in 1910.

This lot comprised 339 insects, 171 males and 168 females. Notes on the duration of life were made on 283 of this number. They emerged between May 8 and June 14.

The 1911 population was tabulated in a correlation table (7) similar to the one for 1910. These emerged at the same time of year as did the late group of 1910, and the duration of life was practically the same. A chance break in the continuity of emergence (fig. 2) is probably due to the drop in temperature during those few days. This is also true for the break of only one day, June 7.

TABLE 7

DATE OF EMERGENCE	DAYS													
	3	4	5	6	7	8	9	10	11	12	13	14		
5-8					1								1	
9							1						1	
10			1	1	2	3		1					8	
11			1		7	1	1		1				11	
12					1		1						2	
13				4	2	1	4	1	1	1			14	
14			1	1	3	2	2	1	1		1		12	
15					2			2	1	1			6	
16							2	2					4	
17						2	2	1					5	
18					1								1	
19		1			1								2	
20														
21														
22														
23														
24						3		2	2	2			9	
25				1									1	
26				1		1	1						3	
27						1	5	1	1				8	
28	1	1		1	1	3	4	2					13	
29				1	1	3		1					6	
30		1	2	2	4	3	2	3	1				18	
31			3	4	4	5	3	6					25	
6-1				1	1	6	2	2					12	
2				2	1	2	7	3					15	
3	2		6	3	12	6		2	2				33	
4	1		5	3	4	2	4	5				1	25	
5			2		2	2	2						8	
6			1	3	1	5					2		12	
7														
8	1	1	1	2	2	1		1					9	
9	1		3		1		1				1		7	
10			1			2							3	
11			3		1	1	1	1					7	
12														
13														
14		2											2	
	6	6	32	29	56	60	41	34	10	4	4	1	283	

In comparing the means for the different classes of this lot (table 1) with that of the late emerging group 1910, we find the duration of life shorter in all cases in 1911. Since this period was warmer in 1911 than in 1910, this only adds one more bit of evidence to our temperature hypothesis. A comparison of table 8 with table 1 will show how in every case a variation in the length of life accords with a simultaneous variation in temperature.

TABLE 8  
*Mean temperatures*

	1909	1910	1911
March.....	44	58	47
April.....	54	56	54
May.....	64	61	71
June.....	75	72	79

Thus the warm March 1910 brought forth the insects at an abnormally early date; a warm May and June 1911 was associated with shorter lives of the animals, while for the same period in 1910, a slightly longer duration of life was associated with the lower temperature.

It will be seen that none of the animals emerged at an abnormally early period and that none lived an unusually long number of days. The table below gives further details on this lot.

TABLE 9  
*St. Louis Cecropias, 1911*

CLASS	DAYS														T.	MEAN
	3	4	5	6	7	8	9	10	11	12	13	14				
Mated ♂'s	1		4	3		4	5	4						21	7.52	
Unmated ♂'s	3	4	8	13	26	24	21	15	5	2				121	7.73	
Mated ♀'s			7	2	10	4	4	1						28	6.96	
Unmated ♀'s	2	2	13	11	20	28	11	14	5	2	4	1	113	7.90		
All mated insects	1		11	5	10	8	9	5					49	7.20		
All unmated insects	5	6	21	24	46	52	32	29	10	4	4	1	234	7.81		
All ♂'s	4	4	12	16	26	28	26	19	5	2			142	7.70		
All ♀'s	2	2	20	13	30	32	15	15	5	2	4	1	141	7.71		
Whole population	6	6	32	29	56	60	41	34	10	4	4	1	283	7.71		

## NEW YORK MATERIAL, CECROPIA 1911

To ascertain just how foreign material would compare with St. Louis material, both in time of emergence and duration of life, 200 *Cecropia* cocoons were procured<sup>14</sup> from Queens County, Long Island, N. Y. These arrived during the latter part of March, and between May 15 and June 3, 139 imagines emerged, 79 males and 60 females. Notes on the duration of life were made

<sup>14</sup> From the American Entomological Company, New York.

col. 133 of these. The mean duration of life of these compares well (table 1) with the data for the St. Louis material for the same year.

TABLE 10  
*New York Cecropias*

CLASS	DAYS														T.	MEAN
	3	4	5	6	7	8	9	10	11	12	13	14				
Mated ♂'s.....			1	1	7	5	4	1		1				20	7.90	
Unmated ♂'s.....		2	1	3	12	6	12	14	5	1				56	8.54	
Mated ♀'s.....	1		2	7	7	2	1	1						21	6.62	
Unmated ♀'s.....	1		2	4	8	4	7	5	3	1			1	36	8.25	
All mated insects.....	1		3	8	14	7	5	2		1				41	7.25	
All unmated insects.....	1	2	3	7	20	10	19	19	8	2			1	92	8.42	
All ♂'s.....			2	2	4	19	41	16	15	5	2			76	8.37	
All ♀'s.....		2	1	11	15	6	8	6	3	1			1	57	7.65	
Whole population.....	2	2	6	15	34	17	24	21	8	3			1	133	8.06	

#### CALLOSAMIA PROMETHEA 1911

To compare the longevity of the *Cecropia* moth with that of others of the same family, some 300 cocoons of *Callosamia promethea* were obtained from Crève Cœur Lake region, St. Louis, early in the spring. These brought forth 183 imagines, 116 males and 67 females. Notes on the duration of life could be made on 170 of this number. It will be noticed (table 1) that the mean duration of life varies greatly in the sexes, and that they do not attain the age reached by the *Cecropias*.

TABLE 11  
*Prometheas*

CLASS	DAYS											T.	MEAN
	2	3	4	5	6	7	8	9	10	11			
Mated ♂'s.....	2	9	6	1							1	19	3.74
Unmated ♂'s.....	6	20	28	24	9	3						90	4.21
Mated ♀'s.....		6	2	5	3	4	1	1				22	5.18
Unmated ♀'s.....	2	2	2	2	8	11	6	6				39	6.54
All mated insects.....	2	15	8	6	3	4	1	1			1	41	4.51
All unmated insects.....	8	22	30	26	17	14	6	6				129	4.91
All ♂'s.....	8	29	34	25	9	3					1	109	4.13
All ♀'s.....	2	8	4	7	11	15	7	7				61	6.21
Whole population.....	10	37	38	32	20	18	7	7			1	170	4.82



Mayer<sup>16</sup> found that most of the *C. promethea* lived about 3 days. Table 11 shows that the life of these insects varied from 2 to 11 days, with the mean falling at 4.8 days. These were kept in confinement; Mayer does not state the source of his data.

The accompanying table correlating the length of life with the time of emergence shows no relation between these two factors.

TABLE 12

DATE OF EMERGENCE	DAYS										
	2	3	4	5	6	7	8	9	10	11	
5-8								1			1
9											
10			2					1			3
11											
12			1								1
13											
14	1	2	2	1							6
15		3	1				1				5
16	1	2	5	2	3			1			14
17		1	2	2	1						6
18		2	4	5	1						12
19		1	3	2	3	3					12
20			1	4	7	2	1				15
21	1		2	5	2	2		1			13
22		3	1	1	1		1				7
23	4	5	2				1				12
24		3	3			2					15
25	2	3	5	2	2			1			8
26		5		1		1		1		1	9
27		3	3	1				1	1		9
28		1		3		3	1				8
29	1					1					2
30				2							2
31		1		1			1	1			4
6-1			1			1					2
2		1									1
3											
4						1					1
5											
6											
7											
8		1									1
9											
10											
11						1					1
	10	37	38	32	20	18	7	7		1	170

<sup>16</sup> Mayer, A. G., *Psyche*, vol. 9, p. 16, 1900.

## TROPAEA LUNA 1911

To gather more data on other species of this family, cocoons of *Tropaea luna* were obtained. Sixty-two imagines (36 males and 26 females) emerged between May 10 and June 4, with one exception on April 23. Records were kept on 60 of these.

It will be seen that the length of life of males and females was almost equal and that the mean for the entire population was greater than that for *C. promethea*. Table 13 gives the details of this work and is self-explanatory.

TABLE 13

*Luna*

CLASS	DAYS									T.	MEAN
	3	4	5	6	7	8	9				
Mated ♂'s.....	1	1	2	1						5	4.60
Unmated ♂'s.....	3	3	3	8	8	3	2	30		6	6.07
Mated ♀'s.....					3	1	1			5	6.60
Unmated ♀'s.....	1	5	4	3	2	4	1	20		5	5.80
All mated insects.....	1	1	2	4	1	1		10		5	5.60
All unmated insects.....	4	8	7	11	10	7	3	50		5	5.96
All ♂'s.....	4	4	5	9	8	3	2	35		4	5.86
All ♀'s.....	1	5	4	6	3	5	1	25		5	5.96
Whole population.....	5	9	9	15	11	8	3	60		5	5.90

## TELEA POLYPHEMUS 1911

Twenty-one cocoons of *T. polyphemus* gave 13 males and 8 females. Notes on longevity were made on 19 of these. None of this species mated in confinement. Table 14 shows a remarkable difference in the life of the sexes, the male varying from 3 to 7 days, and the female from 7 to 12 days. The duration of life of the entire population was 6.79 days.

TABLE 14

*Polyphemus*

CLASS	DAYS												T.	MEAN
	3	4	5	6	7	8	9	10	11	12				
Males.....	2	2	1	4	3							12	5.33	
Females.....					1		4	1		1	7	9	29	
Whole population.....	2	2	1	4	4		4	1		1	19	6	7.9	

## PROPORTION OF SEXES

The accompanying table shows the proportion of sexes of the different lots. The grand total shows the males to be in excess, and especially so in the *Prometheas*. Here the proportion corresponds well to the 111 males and 65 females noted by Mayer in 1899.<sup>16</sup>

TABLE 15  
*Proportion of sexes*

YEAR	SPECIES	MALE	FEMALE	TOTAL
1909	<i>S. cecropia</i> .....	43	25	68
1909	<i>S. cecropia</i> .....	22	13	35
1910	<i>S. cecropia</i> .....	101	104	205
1911	<i>S. cecropia</i> .....	171	168	339
1911	<i>S. cecropia</i> , N. Y.....	79	60	139
1911	<i>S. cecropia</i> , incubator.....	72	90	162
1911	<i>C. promethea</i> .....	116	67	183
1911	<i>T. luna</i> .....	36	26	62
1911	<i>T. polyphemus</i> .....	13	8	21
Total.....		653	561	1214

## PRIORITY OF MALE EMERGENCE

The evidence gleaned from this material substantiates Darwin's conclusion that the males are the first to emerge. In every lot excepting the *Lunas* this was apparent, and in nearly every case where a break in the continuity of emergence occurred, the first subsequently to emerge were also males.

In order to get some tangible proof of this priority, the mean date of emergence was gotten for the males and females of the different species. The difference between these dates would obviously be the mean priority of all of one sex over the other. The differences given in table 16 are in favor of the males in all cases excepting the *Lunas*.

The emergence of the insects on each day is shown in the accompanying curves. Graphs were made for only those species of which we had sufficient numbers to make the curves reliably

<sup>16</sup> *Psyche*, vol. 9, p. 15, 1900.

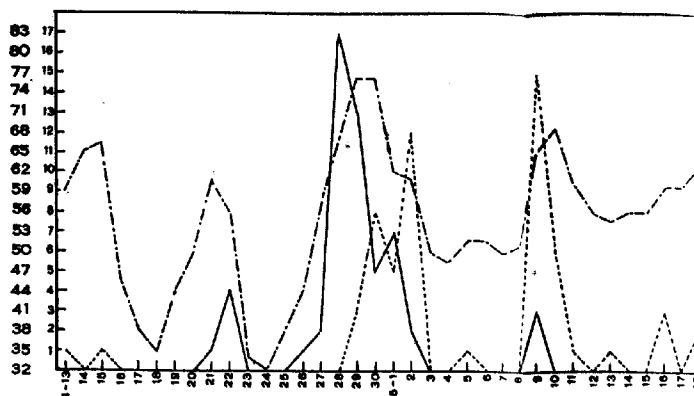


Fig. 1 The emergence of 101 males and 104 females of

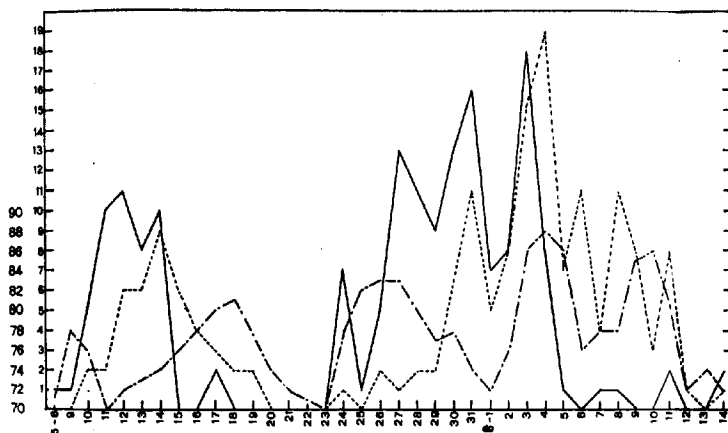


Fig. 2 The emergence of 171 males and 168 females of *Samia cecropia*; St. Louis, 1911 material.

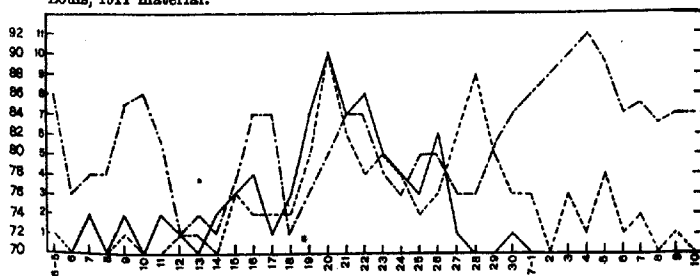


Fig. 4 The emergence of 72 males and 90 females of *Samia cecropia*; incubator material, 1911.



TABLE 16

	Days
Cecropia, 1910.....	1.02
Cecropia, 1911.....	3.85
Cecropia, incubator.....	4.51
Cecropia, New York .....	2.20
Promethea.....	2.68
Polyphemus.....	2.60
Luna.....	1.33

significant. Solid lines are for the males; dotted lines are for the females, and the dash and dot lines represent temperature. The figures on the base line are the dates when emergence occurred, the small numerals on the left are for the number of individuals, and the large figures are degrees of temperature in Fahrenheit. One can see at a glance how the males throughout keep slightly in advance of the females.

An attempt was made to find whether any correlation exists between temperature and rate of emergence. No absolute statement will be ventured upon this point—the data are submitted for the readers' own judgment—but to us it seems highly improbable that such a close agreement as appears in a large part of the data should be no more than coincidence. A careful inspection will reveal a closer agreement than might at first be thought, for of course it must not be expected that at the extremes of the season, when very few individuals emerge, there will be a marked appearance of such a correlation.

In justice to the evidence, however, attention should be called to the fact in the case of those which began to emerge about May 8, 1911, that although the temperature was then only 71°, yet that was a decided leap above the temperature of the preceding week, which was below 55, or about the same as April. The temperature for the balance of the month of May continued abnormally high.

Darwin mentions<sup>17</sup> that this male priority is true of frogs, toads and the majority of salmon, "and throughout the class of insects the males are almost always the first to emerge from the pupal stage." Whether or not this holds throughout the

<sup>17</sup> Descent of Man, p. 240. A. L. Burt's reprint from 2 ed. n. d.

class of insects does not seem to be fully known, but in this material it is clearly evident.

An explanation of this early male emergence was attempted by Darwin. "The cause of the difference between the males and females in the period of arrival at maturity is sufficiently obvious. Those males which annually first migrated into any country, or which in the spring were first ready to breed, or the most eager, would leave the largest number of offspring and these would tend to inherit similar instincts and constitutions."

This explanation is far from clear when we try to apply it to Saturniid material. A more probable explanation would be that the males emerge first merely because they do not require such a long period of development to mature their gonads. The female has a large number of ova to nourish, probably requires a greater amount of food in the larval stage and a greater period of pupation, and late emergence follows.

But the item of interest is to find whether early emergence of the males is an adaptation, as Darwin seemed to think, for the benefit of the species. From the data of 1910 it seems that 'adaptation' gives us males at one time, and when these are almost dead or too old to mate then a succession of females, many of which die without leaving issue owing to the lack of mates. To illustrate this let us say that with 101 good males and 104 females in 1910, 52 males (more than one half) could not be mated, and even though we called to aid some of the 69 stray males which were attracted to the laboratory, still it was possible to supply only 65 of the females with mates. It would seem that for the benefit of the race both sexes should emerge at about the same time (since both are mature for mating at about the same age) in order to eliminate any expenditure of energy in finding each other, and to eliminate any possibility of a great proportion of the males and females dying without having mated, in spite of the fact that the sexes may be equal.<sup>18</sup>

It was suggested that the early maturity of the male may be due to a shortened period of development. This is deserving of

<sup>18</sup> It may be that the males of one area migrate to another territory to accomplish mating, but we have no proof to that effect.

careful investigation on the length of pupal, larval and egg stages of both sexes, and might lead to a simple and proper explanation of this phenomenon.

#### COMPARISON OF THE LENGTH OF LIFE OF THE SEXES

In 1909 it was found that the males of the *Cecropia* were longer lived than the females. In comparing the duration of life of the sexes in table 1 we find the males to have lived longer in the 1910 *Cecropias*, the New York, and the Incubator *Cecropias*. In the Lunas and the 1911 St. Louis *Cecropias* there was not a significant difference. In the *Polyphemus* and *Prometheas*, the opposite is true; we find a great difference in favor of the females.

#### COMPARISON OF THE LENGTH OF LIFE OF MATED AND UNMATED INSECTS

In the entire *Cecropia* material and also the *Prometheas*, we find no appreciable difference in the length of life of the mated and unmated males. In the females of the six lots, however, we see a significant difference apparently resulting from this condition, the unmated females being the longer lived. It is very interesting that mating may be such a tax upon the females (it cannot be the ovipositing, for the unmated females also experience that) as to effect a curtailment of life. No such curtailment due to mating is detected in the males. In the Lunas, we find a very slight difference in favor of the mated females. This result, however, is derived from a small number. Inspection of the two columns of table 1, all mated and all unmated insects, shows that the balance swings in *every* case toward longer lives in the unmated insects.

#### LAPSE OF TIME BETWEEN LAST EGG LAYING AND DEATH

According to Weismann's theory one would expect that in a monogamous species the males would die soon after mating, while the females would live long enough to completely oviposit.

In the five lots of *Cecropias* we find the mean duration of life to be even greater in the mated males than in the mated females. Surely the continuation of such a long, useless life in the male cannot be an adaptation for the good of the species. In both



the 52 fertilized and the 28 unfertilized females of 1910 we found<sup>19</sup> that life was cut short while most of the insects retained many eggs. Now if the duration of life be an adaptation for the good of the species, why were not such lives permitted to continue? In many cases also a lapse of time, sufficient for completing oviposition, intervened between the last egg-laying and death. Tables 17 and 18 shows the number of eggs each female retained and the number of hours it lived after ceasing to oviposit.<sup>20</sup>

TABLE 17

*Fertilized females*

EGGS	HOURS	EGGS	HOURS	EGGS	HOURS	EGGS	HOURS
240	0	41	16	12	14	3	0
165	8	41	7	10	0	3	30
151	0	34	0	9	40	2	0
119	15	31	14	9	?	1	7
105	7	29	14	8	0	1	15
101	16	27	6	8	38	1	14
95	38	22	7	8	?	0	96
94	6	20	0	7	?	0	15
93	60	20	0	6	?	0	4
80	17	18	15	5	41	0	39
72	0	15	7	5	0	0	8
56	12	14	38	5	12	0	38
54	0	13	15	3	24	0	?

TABLE 18

*Unfertilized females*

EGGS	HOURS	EGGS	HOURS	EGGS	HOURS
270	?	103	14	40	2
257	14	102	?	37	0
247	?	100	?	32	24
189	12	85	6	31	30
179	0	80	0	23	?
144	7	68	0	21	0
137	6	62	?	17	?
135	?	53	0	2	?
133	10	48	36	2	?
110	?				

Trans. Acad. Sci., St. Louis, vol. 20, p. 314-315, 1911.

Less than six hours is designated by 0.

Here we may see many insects, both mated and unmated, dying in the very midst of egg-laying. Then again we see others which perfectly or almost perfectly oviposited, continuing a useless life in some cases up to 38 or even to 96 hours.

The long life of some females after completely ovipositing, and the duration of life of others to insufficient complete egg-laying, and the long useless life of the male all lead to the belief that the duration of life is not, as Weismann says, an adaptation for the good of the species which came about through Natural Selection.

It seems more natural to assume that the duration of life depends upon the amount of reserve nutriment which the insects acquire at an earlier stage, the activity of the insects, and the climatic conditions. The climatic conditions seem to influence the insects' activity; the activity effects the expenditure of reserve nutriment, and this expenditure controls the length of life, for the nutrition of the imago depends wholly upon this reserve. In short, it seems that the length of life depends to a degree upon physiological processes.

In 1911 data were gathered on the completeness of oviposition and the lapse of time between last egg-laying and death in *C. promethea*,<sup>21</sup> and the same facts were found to hold for this species as for *S. cecropia*. In *Promethea* it was not definitely ascertained whether or not they are monogamous.

#### THE RELATION OF LONGEVITY TO THE REPRODUCTIVE FUNCTION

Weismann says: "No better arrangement for the maintenance of the species . . . can be imagined than that supplied by diminishing the duration of life and simultaneously increasing the rapidity of reproduction." If this were true of the *Cecropia* moth we should find this monogamous species living only long enough to carry on the function of reproduction, i.e., males dying soon after mating and the females living long enough completely to oviposit. This we by no means find. Table 1 shows that the males live about as long as the females, even though they are of no further use to the species. We have shown that among the

<sup>21</sup> Details are in course of preparation for publication.

females some lived a considerable time after all eggs were deposited, others died retaining many eggs although they had ample time to completely oviposit, and still others died in the midst of ovipositing. In 1910 it was found that there was absolutely no relation between completeness of oviposition and (1) long life, (2) the longer or shorter time spent in copulo, or (3) the age of the insects at mating. The mated females, however, oviposited more completely than the unmated ones, although they had less time to devote to it owing to the long time spent in mating.

In considering whether or not the length of life is an adaptation for the good of the species, the completeness of oviposition seems to be a factor worthy of attention. The discoveries in the relation of long life to completeness of oviposition, as well as the long, useless life of the males, do not seem to substantiate this theory.

#### CONCLUSION

When the facts gleaned from these observations are compared with the various theories which have been advanced to account for the duration of life, one becomes conscious of the fact that much direct work must be done before any broad generalizations can be applied. Very little is accurately known of the normal ages of the different members of the animal and vegetable kingdoms, and still less on the relation of longevity to reproduction—why it is that one organism lives for a certain period while another, which may mature in the same length of time, or may attain the same size, or live in the same environment, attains an entirely different age.

Weismann, as we have already stated, thinks that the duration of life is regulated solely by the needs of the species, and that this came about through natural selection. Eimer hints about the inheritance of acquired characters. Morgan seems to think that the problem of the duration of life is one for physiological investigation, but goes on cautiously to mention the mutation theory. Thus it continues, each man finding in the duration of life a conformity to his own already formulated theories. Whether it is a subject to be considered in any theory of evolution

will be known only after much work has been done upon the relation of longevity to the function of reproduction in a vast variety of species.

Our work leads us to believe that there are still factors unknown which influence the duration of life. One of these, the influence of climatic conditions upon the length of life in the *Cecropia* moth, we have been fortunate to discover. If it were only adverse conditions curtailing life, no great significance could be attached to it, but when favorable, though abnormal, conditions were discovered and supplied, life and activity continued far beyond what was thought to be possible. This has hitherto not been considered in any of the theories, and the experimental work here recorded shows this factor to be of importance. There are no doubt many other influences awaiting discovery, which will have to be considered before we dare philosophize upon the subject.

It would be well to suggest some of the phenomena yet to be investigated upon a wide variety of material before a theory can be firmly established to account for the duration of life.

1. The difference in the duration of life of the sexes.
2. The duration of life of mated and unmated individuals of both sexes.
3. The proportionate numbers of the sexes.
4. The maturing of one sex before the other.
5. Monogamy or polygamy.
6. The relation of longevity of the female to the welfare of the young. In some forms where the parents do not care for the young, is the duration of life sufficient for bringing forth all the young (e.g., complete oviposition or seed-bearing), and in those which care for the offspring, is the length of life adequate for the necessary care of the young (e.g., food and flying in birds)?
7. Number of potential ova that the organism may possess when overtaken by death.
8. Does old age cause the inability of the individual to bring forth the young, although sexually capable? (e.g., lapse of time after incomplete oviposition in the *Cecropia*).
9. What proportion of the eggs deposited by a mated female are fertile?

10. In animals (similar to those considered in this paper), in which the female is fertilized once for all, is there any relation between time spent in copulo and the fertility of the eggs; i.e., does a long period of copulation insure the fertility of all the eggs?

11. Is copulation itself correlated with longer or shorter life?

12. The effects of climate.

13. Food conditions of the adult; its effect upon the present and the subsequent generations.

14. Nutrition and environment in developmental stages.

These ideas, which make no claim of completeness, were gleaned principally from work with these insects, hence many are inadequate or cannot apply to other forms. It is true that many organisms will not permit of such investigation, and that confinement in some cases will cause marked changes. It may be years before sufficient and conclusive data can be had for the solution of the problem, but the vital importance of the phenomena of longevity in relation to the interests of the human race, as well as biologically, should at once arouse investigators to the accumulation of such data, even though it be only as by-products to their chosen line of work.

St. Louis, Mo., October 30, 1911.

# OBSERVATIONS ON THE ORIGIN AND SEQUENCE OF THE PROTOZOAN FAUNA OF HAY INFUSIONS

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FIFTEEN FIGURES

## CONTENTS

i. General introduction.....	206
ii. Origin of the protozoan fauna of hay infusions.....	207
A. Experiments.....	208
B. Results.....	210
C. Conclusions.....	213
iii. Relative number and sequence of representative protozoan forms in hay infusions.....	213
A. Experiments.....	215
B. General observations on the course of development of hay infusions.....	222
C. The A, B and C groups of infusions.....	224
D. Time of appearance, maximum number and disappearance of representative protozoan forms at the surface of the infusions.....	225
1. Monad.....	225
2. Colpoda.....	228
3. Hypotrichida.....	231
4. Paramaccium.....	234
5. Vorticella.....	236
6. Amoeba.....	238
iv. Summary of surface observations.....	241
v. Protozoan fauna at the middle of the infusions.....	244
vi. Protozoan fauna at the bottom of the infusions.....	246
1. A Infusions.....	246
2. B Infusions.....	247
3. C Infusions.....	250
vii. Discussion and conclusions from the observations on the sequence of the surface, middle and bottom fauna.....	250
1. Surface fauna.....	250
2. Middle fauna.....	254
3. Bottom fauna.....	254
4. Factors determining the sequence.....	256
5. Decline in numbers.....	259
viii. Conjugation.....	260
ix. Summary.....	262

## I. GENERAL INTRODUCTION

Although hay infusions have been one of the chief means of providing organisms for microscopists from the early days of Leeuwenhoek, there are comparatively few published data which have been secured through a careful study of the origin, relative number, and sequence of the various organisms which abound in them. It is a well known fact that a hay infusion presents a kaleidoscopic series of phenomena from its inception until it finally reaches a stage of sterility, or, in the presence of sunlight, of practically stable equilibrium in which animals and green plants become so adjusted that a veritable microcosm exists; and it is also generally accepted, largely on the basis of casual observation of infusions made up for one purpose or another, that the organisms appear and disappear in quite a regular sequence.

It seemed desirable, accordingly, to attempt to study the fauna and flora of representative infusions by some comparatively exact methods. The first intention was to make a comprehensive tabulation of the entire animal and plant life of the infusions studied, including bacterial counts, as well as to follow the chemical and physical changes in the medium. This proved to be impossible without the aid of more assistance than was available.<sup>1</sup> Consequently the study was chiefly confined to a careful observation of the Protozoa which appeared, and especially to certain characteristic forms which were present in large numbers in practically all the infusions studied.

This general problem has been considered by Peters,<sup>2</sup> but more data were needed for points of attack on the biological effects of one type of organism on another, and this survey of the protozoan

<sup>1</sup> Certain chemical analyses, chiefly in regard to the acidity of the infusions, were made by Dr. M. S. Fine, and his results are published independently in the following paper, in this journal, entitled: Chemical Properties of Hay Infusions with Special Reference to the Titratable Acidity and its Relation to the Protozoan Sequence.

<sup>2</sup> Metabolism and division in Protozoa. *Proc. Amer. Acad. Arts. and Sci.*, vol. 39, no. 20, 1904. Chemical studies on the cell and its medium. 1. Methods for the study of liquid culture media. *Amer. Journ. Physiol.*, vol. 17, no. 5, 1907; 11. Some chemico-biological relations in liquid culture media. *Amer. Jour. Physiol.*, vol. 18, no. 3, 1907.

fauna of hay infusions is preliminary to further studies on the interactions of particular species on each other.

## II. ORIGIN OF THE PROTOZOAN FAUNA OF HAY INFUSIONS

The point first considered was the source or sources from which the protozoa which appear in infusions of hay are derived. This general problem has, of course, been treated at length in the long series of experiments on spontaneous generation which occupied the attention of biologists for several centuries. The present experiments were planned to determine the best method of making up infusions for the purpose of the study of their biological cycle, and incidentally to show the relative importance of air, water and hay, and whether some forms appear in infusions chiefly through one of these channels and others through another.

Hay is generally considered the chief source of the protozoan life of infusions. Kent<sup>2</sup> in 1879 studied the question

from whence (are) derived all these myriad organisms frequently produced in such abundance as to literally jostle each other for room in every drop of water extracted for examination? . . . . . hay from different localities was placed in maceration and examined continuously from its first contact with the fluid medium, from periods varying in duration from a few days only to several weeks. The water added to the hay was of the purest possible description, and was frequently boiled for some time to prevent the introduction of extraneous germs. In all instances, the results obtained were broadly and fundamentally the same, and differed only with respect to the specific types found living together in the separate infusions. Even here, however, the general dominance of two or more special forms was notably apparent.

Kent was satisfied, then, that the organisms were derived from the hay, and microscopical examination of the mode of distribution of the cysts upon the lowermost blades, colored brown or yellow from incipient decay, led him to conclude that "all the essential conditions of their life cycle had been passed in close connection with it." He put this conclusion to a practical test by gathering grass saturated with dew during a heavy fog and studying it without the addition of any water.

<sup>2</sup> A manual of the infusoria. London, 1880, pp. 135-141.



In every drop of water examined, squeezed from the grass or obtained by its simple application to the glass slide, animalcules in their most active condition were found to be literally swarming . . . . . Their purpose in life, as in the case of the animalcules inhabiting artificial infusions, is to break down and convert into new protoplasmic matter this otherwise waste product . . . . . To maintain the balance here, however, and to check the too rapid increase of the various herbivorous monads, we find other types . . . . . developed side by side with and feeding in turn upon the plant-eating species.

Following Kent's method, I have examined grass from the campus wet with dew and light rain, and have obtained substantially the same results. Active forms of various flagellates, chiefly monads, and ciliates such as Colpoda, Chilodon, etc., were observed swimming in the moisture on the blades of grass. I have not found them in such great abundance as described by Kent, but still in sufficient numbers to make an interesting demonstration. Goodey, also, in his recent study of the Protozoa of the soil found a number of active forms among the surface vegetation.<sup>4</sup>

#### A. EXPERIMENTS

Twenty-four hay infusions were made up, and kept in a well lighted room in the laboratory, at room temperature. Twelve contained hay cut near the laboratory, and twelve practically pure timothy hay from a farm near New Haven. These two sorts of hay were designated, for convenience, Y and T respectively. Each infusion consisted of about 5 grams of hay in 1 liter of tap water, and was contained in a flask with a capacity of 1500 cc. These infusions were divided into four groups of from four to eight infusions, and in each of these four groups, half of the flasks contained Y, and half T hay. The four groups of infusions were designated by the letters, A, W, H and WH respectively.

*A Infusions.* In this group of eight infusions the hay was put into the various flasks and then subjected to seven pounds pressure of steam for one hour in an autoclave. The water was likewise subjected to the same conditions and when it had again

<sup>4</sup> A contribution to our knowledge of the Protozoa of the soil. Proc. Royal Society, Series B, vol. 84, 1911.

reached the room temperature it was added to the sterile hay in the flasks. Four of these infusions were left exposed to the air, and four were plugged with cotton, sterilized dry at a temperature of 180° C. for one hour.

*W Infusions.* The hay in these six infusions was sterilized exactly as in the case of the *A* series, but the water was not sterilized. All the flasks were plugged with sterile cotton.

*H Infusions.* The water used in this series of six infusions was sterilized as in the case of the *A* series, and to this water was added fresh hay. Sterile cotton plugs were inserted in each flask.

*WH Infusions.* These infusions, four in number, were made by simply adding fresh hay to ordinary tap water. The tops of the flasks were covered with inverted beakers.

The twenty-three infusions may be tabulated as follows:

*A Series*—to determine the organisms derived from the atmosphere.

*At1, At2, Ay1, Ay2* = sterilized hay and water. Exposed to the air.

*At3* = control; sterilized hay and water. Not exposed to the air, but plugged with cotton.

*At4, Ay4* = control; sterilized hay and water. Inoculated with pure cultures of *Paramecium aurelia* and *caudatum*, and also with *Oikomonas*. Plugged.

*W Series*—to determine the organisms derived from tap water.

*Wt1, Wt2, Wt3, Wy1, Wy2, Wy3* = sterilized hay and fresh water. All the flasks plugged with sterile cotton.

*H Series*—to determine the organisms derived from the hay.

*Ht1, Ht2, Ht3, Hy1, Hy2, Hy3* = sterilized water and fresh hay. Flasks plugged.

*WH Series*—control.

*WHt1, WHt2, WHy1, WHy2* = fresh water and fresh hay. Mouth of flask covered with inverted beaker.

The experiments were started on July 29th. The infusions were examined at intervals of approximately one week during the following six weeks, and at irregular intervals thereafter until November 11th, when the remaining infusions were destroyed. It was planned to carry the observations for six weeks, but at the end of this time it seemed advisable to continue certain ones longer.

## B. RESULTS

*A Series.* The *A* series gives evidence as to the general influence of the atmosphere as a source of protozoan life in laboratory infusions. The mouth of the containing flasks measured  $1\frac{1}{2}$  inches in diameter and thus afforded ample exposure to the air without rendering rapid evaporation troublesome. The flasks stood during most of the time in a room in which hay was being used for various purposes, and consequently there was ample opportunity for the air to be contaminated with cysts, etc. Also, during the day time the windows at either end of the room allowed a considerable current of air to pass over the flasks. Again, certain flasks were placed on a shelf outside of the window where they were exposed to the air of the campus.

The results derived from this series are as follows: *Ay1* remained free from protozoa from the start to October 31st, at which time it was seeded with *Paramaccium* and *Oikomonas*. Two days later it showed a good growth of each of these forms, thus proving that it was a favorable fluid for protozoa. *Ay2* was sterile in regard to protozoa until September 24th when a very few tiny amoebae appeared, and remained until October 31st, when the culture was destroyed. *At1* contained on August 26th a few small hyaline bodies which seemed to be cysts. A week later there appeared a few tiny amoebae, and on September 24th a heavy growth of monads was observed which persisted to the discontinuance of the infusion on October 31st. *At2* remained sterile until September 24th when tiny amoebae appeared and continued to be present to the end. On October 31st the infusion was seeded with paramaccia and these had greatly increased in number by November 2nd when the infusion was destroyed. *At3*, which was kept plugged as a control, was first examined on September 2nd and was sterile. It was discontinued at this time. The *At4* and *Ay4* cultures were seeded at the beginning with *Paramaccium* and *Oikomonas* and showed heavy growths from the start—thus proving that the media, from the inception of the experiments, offered favorable conditions for protozoan life. These two infusions were discontinued on November 2nd.

It is believed that in this series of experiments exceptional opportunities were offered for infection of the culture medium by air borne cysts, etc., to occur, and the resulting protozoan fauna shows that the atmosphere is a negligible factor in the seeding of hay infusions used for laboratory study.

*W series.* The data from the *W* group of infusions show the protozoan life which was introduced with the laboratory tap water. *Wy1* showed from the start heavy growths of *Chilomonas*, *Oikomonas*, and *Chilodon*, and these persisted in varying numbers until November 9th. At this time the culture was seeded with paramaecia and two days later there was a considerable increase in their number. The culture was discontinued at this time. In the *Wy2* infusion there appeared several species of monads, including *Oikomonas* and *Bodo*. A rotifer (*Rotifer vulgaris*) was observed on August 12th and increased in numbers until there were about 2000 per cc. at the top of the infusion, when the culture was discontinued on November 11th. The culture was seeded toward the end with paramaecia which multiplied rapidly. *Wt1* and *Wt2* developed numerous species of monads and also considerable growths of a tiny amoeba. *Wt1* had as many as 5000 per cc. when it was lost by an accident on September 2nd. *Wt2* on the same day had 20,000 amoebae per cc., and on September 24th these were succeeded by myriads of *Amoeba radiosa*. The culture was seeded with paramaecia on November 9th, and was destroyed on November 11th when it contained a good culture of this animal. *Wy3* and *Wt3* remained plugged, as a control, until November 9th and when examined on this day they contained practically the same fauna as the other cultures of the *W* series as described above. A point worthy of special note, however, is that *Wt3* showed, in addition to many tiny amoebae, about twenty-five *Amoeba proteus* per cc. of the fluid at the top of the culture. It is interesting that in certain cultures heavy growths of tiny amoebae appeared; that in one culture these gave place to *radiosa* forms; and in a third, *Amoeba proteus* appeared. This suggests the possibility that *Amoeba proteus* was introduced in the form of extremely minute spores which became apparent as tiny amoebae, later became amoebae of the *radiosa* type, and in

one culture, developed as far as the typical proteus form (cf. p. 255).

Obviously tap water will vary from time to time throughout the year, and no emphasis is placed on the completeness of the experiment in respect to the species which can be introduced through this channel. However, the work is extensive enough to clearly show that an insufficient number of species of Protozoa is introduced with ordinary tap water to make this a practical method for seeding infusions for study.

*H Series.* The organisms which appeared in these cultures must have been encysted on the dry hay with which the infusions were made, and therefore they represent at least some of the forms which one may secure in the laboratory through this source. *Hy1*, *Hy2*, *Ht1*, and *Ht2* showed a closely similar series of forms, including all those which have been noted in the previously described cultures except *Chilomonas* and typical *Amoeba proteus*, and in addition several species of *Colpidium*, *Colpoda*, *Oxytricha*, and other hypotrichous forms, *Glaucoma*, *Holophrya*, *Spathidium*, *Bursaria*, etc. All of these infusions were seeded with *Paramaecium* on November 9th, and when discontinued there was a heavy growth of this organism in each, thus proving that a favorable medium was present for *Paramaecium*. *Hy3* and *Ht3*, served as a control, and were not examined until the end of the experiments when they contained essentially the same forms as the other members of the *H* series.

*WH Series.* This group of infusions, consisting of fresh hay and water partially exposed to the atmosphere, was carried as a control for the above experiments. The protozoan fauna which developed was somewhat more meager than that developed by the *H* series. The explanation of this fact is not at once apparent since the hay and the water employed came from the same source as that used in making the other infusions. It was evident that the cycle of the infusions of this series developed more rapidly than those of the other series, and a possible explanation is that the bacteria introduced with the water so augmented the initial processes of decay with their attendant phenomena that a medium less favorable for large growths of various protozoan forms was

produced. These data, though too meager to be conclusive, suggest that sterile water added to fresh hay may prove to be a better medium for the development of the protozoa encysted on the hay.

### C. CONCLUSIONS

Viewed in their entirety, these twenty-three infusions indicate that: (1) Ordinary hay added to tap water usually will not produce an infusion which is productive of a sufficient number of representative forms to make it profitable for a study of protozoan sequence. (2) Air, water, and hay are all sources from which the Protozoa are derived, and increase in importance in the order given. Of these three, however, air is practically a negligible factor in seeding infusions.

### III. RELATIVE NUMBER AND SEQUENCE OF REPRESENTATIVE PROTOZOAN FORMS IN HAY INFUSIONS

A somewhat regular sequence of organisms in infusions of one kind or another attracted the attention of the early devotees of the simple microscope, as is shown, for example, by the following paragraph from a letter written in September, 1702, by an anonymous person who was led by the writings of Leeuwenhoek to make such studies:

In my observations of the *Animalcula in Waters* I have seen many of the same species in the several infusions, and even in Waters that have been exposed (especially at this time of the year) any time without any particular mixture, such as you find in the hollow of a Cabbage-leaf, or on the *Dipsacus*, etc., and I am confident that many of these are the same Creatures under different dresses. For I have noted such a regular process in them, and such a constant order of their appearance, that I am of opinion most of them are the product of the Spawn of some invisible *Volatile Parents*<sup>5</sup> . . . .

Nearly a century and a half later Dujardin, from his experience with infusions, wrote:

<sup>5</sup> Philosophical transactions, Royal Society, London, vol. 23, 284, 1703, p. 1366. This communication is accompanied by the first published figure of *Paramecium*. From the description in the text, however, it is evident that the author at times confused *Paramecium* and certain hypotrichous forms. These same figures are reproduced by Baker, in his treatises on the microscope.

Depuis l'instant de sa préparation, une infusion change incessamment, et plus ou moins vite, suivant la température; elle montre seulement d'abord le *Bacterium termo*, puis quelqu'autre *Bacterium* et le *Vibrio* linéole, puis des Monades, des Amibes et quelques autres *Vibrions* ou *Spirillum*; un peu plus tard, les *Enehelys* et les *Trichodes* commencent à s'y montrer avec des *Kolpodes* qui, grossissant rapidement, se montrent conformes au type nommé *Kolpoda cucullus*; enfin, viennent les *Trachelius*, les *Loxodes*, les *Coccudina* ou *Ploesconia*, les *Paramécies*, les *Kérones*, les *Glaucomes* et les *Vorticelles*, soit tous ensemble, soit séparément; mais toujours à peu près des mêmes animalcules, de ceux que Joblot nommait d'une manière très-significative les *Cornemuses*, les petites *Huitres*, les *Chaussons*, que *Gleichen* appelait les *gros et petits Ovaies*, les *Pendeloques* et les animalcules *pantouffles*. Le nombre en est assez restreint, et c'est à peine si les quinze genres que nous venons de citer fournissent en tout quarante ou cinquante espèces. Si les infusions sont conservées pendant longtemps, elles changent tout à fait de nature; pourvu que le liquide soit en quantité suffisante, la substance mise à infuser devient un sol sur lequel peuvent se développer des végétations, ainsi que sur la paroi du vase; si la lumière est assez intense, on observe même des végétations vertes; alors, avec d'autres Infusoires on peut rencontrer dans les liquides des *Systolides* et des *Diatomées*.<sup>6</sup>

It is obvious, however, from the preliminary experiments outlined in this paper in regard to the origin of protozoan fauna of hay infusions, that the Protozoa which appear, when laboratory water is added to ordinary hay, are insufficient in variety to render their study profitable from the standpoint of the sequence of forms, because, to determine a sequence of any general interest, it is necessary that a large number of species be present initially so that the dominating forms may be selected for particular study. It would clearly be easier to work out the sequence of forms encysted on the hay, but by doing this a sequence would be obtained which would represent merely that of a special group of forms and this would obviously vary more or less with each lot of hay. Again, since paramaccia cannot be secured from dried grass, this form would not appear in the series.

It was necessary then to employ other means of making up and seeding the infusions, so that there would be no doubt but that all the more common protozoan forms were present at the beginning. It was also necessary to start as many infusions as could be carefully studied simultaneously, in order to have the record

<sup>6</sup> Histoire naturelle des Zoophytes. Infusoires. Paris, 1841, pp. 173-174.

sufficiently comprehensive to rule out as far as possible individual variations and give final results of some general applicability; for, as Dujardin quaintly expressed his own experience with infusions:<sup>7</sup>

Rien de plus simple que de préparer des infusions et d'y voir se produire les Infusoires; mais rien de plus difficile que d'obtenir des résultats semblables de deux infusions préparées en apparence dans les mêmes conditions: c'est qu'en effet les circonstances ne peuvent jamais être exactement semblables. En supposant que la dose des ingrédients et la qualité de ces ingrédients soient les mêmes, la température, l'état hygrométrique et l'état électrique, ainsi que l'éclairage, et l'agitation ou le renouvellement de l'air, n'auront pas pu être les mêmes ou varier de la même manière dans les deux cas. Or, toutes les causes exercent sur le développement des Infusoires une influence qui, pour n'être pas scientifiquement déterminée, n'en est pas moins bien réelle et souvent bien considérable.

#### A. EXPERIMENTS

Twenty-six infusions were made up with nearly pure timothy hay and laboratory tap water. In every case 20 grams of hay and 5 liters of water were put into a glass battery jar with a capacity of about  $5\frac{1}{2}$  liters. Each was loosely covered with a plate of glass to prevent undue evaporation and the entrance of dust. The jars were situated in a small room with windows on three sides so that all the infusions received practically the same illumination. The temperature was recorded with a maximum and minimum thermometer. With this as the general plan, three methods of procedure were followed, giving three types of infusions designated respectively, *A*, *B* and *C*.

*A Infusions.* In this series the hay was boiled for five minutes in approximately 250 cc. of water and then sufficient tap water was added to make 5 liters. This infusion was then 'seeded' with 5 cc. of material from laboratory infusions and aquaria rich in animal and plant life. The 'seed' used in this series and in the following *B* series was thoroughly mixed in a flask before being added, so that each was seeded as nearly the same as possible.

*B Infusions.* These were made up exactly the same as the *A* series, except that the hay was removed from the infusion by

<sup>7</sup> Loc. cit., pp. 170-171.



straining it through cheese cloth. This eliminated all but an insignificant number of the smallest fragments.

*C Infusions.* To make up this set, 20 grams of hay was put into five liters of tap water. It was neither boiled nor strained. A few drops of 'seed' was added, thus insuring the presence of all the chief forms seeded into the *A* and *B* infusions.

The twenty-six infusions were made up at intervals and were designated as follows:

- April 1st: A-1, A-2, A-3, A-4, A-5, A-6, B-1, B-2, C-1, C-2, and C-3.
- April 13th: A-21, A-22, B-21, and B-22.
- April 24th: A-31, A-32, B-31, B-32, and C-31.
- May 1st: A-41, A-42, B-41, B-42, C-41, and C-42.

Each of the infusions existing during April was studied daily from its inception to May 1st. After this date the observations were made for a while at forty-eight hour intervals, and then at somewhat longer intervals depending on the rapidity of change in the respective cultures. The last regular count was made on June 26th, 1909, but since that time up to the present (Oct., 1911) the infusions have been kept under general observation.

The methods of study consisted of an examination of samples of the liquid taken from the top, middle and bottom of the jars, and the enumeration of the different Protozoa, Rotifera, Algae, etc., which were present. The liquid was removed from the jar for study with a 5 cc. pipet. The 'surface' medium studied was taken from three points in the jar just under the surface film; one at the side nearest to the chief source of light, another at the side farthest from the chief source of light, and the third directly at the centre of the surface of the infusion. The 'middle' medium was taken from this portion of the infusion by inserting the point of the pipet quickly to the region, while the other end of the pipet was closed with the finger. The 'bottom' medium was taken in a similar manner. In 'middle' and 'bottom' counts care was exercised to move the tip of the pipet through the respective regions in order to get a representative sample. Only one pipetful was taken in each of these counts because of the possibility that a few organisms might get into the pipet when it was passing through the upper portion of the fluid on its downward

course, and such error as existed from this would only be augmented by passing the pipet more than once through this region. Various methods were tried to avoid this error entirely. For example, when the study of a sample suggested that possibly some of the organisms observed might have entered from the surface fluid, another sample was taken with a pipet in the tip of which a cork was inserted. When the pipet in this condition had reached the point from which the sample was desired, a wire was inserted through the pipet and the cork pushed out. The pipet, of course, immediately filled with water up to the level of the surrounding infusion and the cork itself rose to the surface. In the great majority of cases it was found that samples taken by this latter method simply corroborated those taken by the more expeditious means, and consequently it is believed that the data secured with the method generally used in the work possesses an error which is negligible.

After a sample of the infusion had been removed it was immediately put into a watch glass and stirred, and then 1 cc. was taken with a pipet and put into a Sedgwick-Rafter counting cell. As is well known, this consists of a glass slide upon which is cemented a metal rectangle. The dimensions of the space enclosed by the rectangle is 50 x 20 mm., and, as the metal is 1 mm. thick, when the rectangle supports a large cover glass it forms a cell which has a capacity of exactly 1 cc. The sample to be examined, then, was spread out on the slide to a depth of 1 mm., and presented to view a total of 1000 cubic mm. The contents of this cell was then at once examined under a microscope which was provided with an ocular micrometer so ruled that, with lenses and tube length properly adjusted, a square of the micrometer just covered 1 sq. mm. of the field, and by focussing through the depth of the liquid enclosed by the square, a volume of the sample equal to 1 cu. mm. was under observation. By counting the organisms which were included, during a unit of time, in the 1 cu. mm. under observation, and multiplying this by 1000, the number of organisms in the cell could be ascertained.<sup>3</sup> Usually ten such counts,

<sup>3</sup> For a detailed description of the apparatus, cf. Whipple: *The microscopy of drinking water*, 2d ed. 1910.

each of about one minute duration, were made for each sample and their average taken. This was the general method of observation employed, but in samples in which only a few comparatively large forms were present the number of each species was counted directly under a dissecting lens. Again, in cases in which myriads of the tiniest active monads were present it was impossible to count them satisfactorily and accordingly it was necessary to estimate the number present on the basis of the experience gained by the use of the exact counting system. In addition to the observations made with the compound microscope, in nearly every case the sample was also examined with a lens magnifying about ten diameters, in order that a comprehensive view of the slide could be secured which would serve to indicate the general distribution of the organisms on the slide, and act as a check on the more exact observations.

Accordingly, while the enumeration of the organisms varied as exigencies demanded, all the counts were made by one person and consequently the personal equation of the observer, which must influence to some extent the data collected from such a series of observations, remained the same. It is believed that the data secured are sufficiently comprehensive to give accurately the relative number and to show approximately the actual number of the various organisms present. It is obvious, of course, that the method employed does not give data which show the presence in the infusions of one or a dozen organisms. Therefore the terms employed, 'time of appearance' and 'time of disappearance,' indicate simply the presence or absence of a sufficient number of animals to be detected by the method. More than this, I believe, could not be secured without the expenditure of more labor than one individual could devote to it daily for a period of three months.

Obviously the rate of development of an infusion will depend upon the temperature to which it is subjected, and, within limits, the higher the temperature the more rapidly the sequence of forms will proceed.<sup>9</sup> The ideal way, therefore, to conduct such a series of experiments as these under consideration would be to

<sup>9</sup> Woodruff and Baitzell: The temperature coefficient of the rate of reproduction of *Paramecium aurelia*, *Am. Jour. Physiol.*, vol. 29, no. 2, 1911.

maintain a constant temperature throughout the work. This was impracticable when the observations were made and considerable fluctuations in temperature occurred. However, all the infusions of the same set were subjected to the same temperature and consequently the relative time of appearance of the different forms in these is directly comparable. As the work progressed, from April to June, the average temperature of the room increased (cf. table 1), and consequently the infusions made later than April 1st, were subjected from the start to higher temperatures than the former. Thus it is impossible to compare accurately the con-

TABLE 1

DATE, 1909	TEMPERATURE (F.)		DATE, 1909	TEMPERATURE (F.)		DATE, 1909	TEMPERATURE (F.)	
	MAXIMUM	MINIMUM		MAXIMUM	MINIMUM		MAXIMUM	MINIMUM
April 4	52	49	April 29	76	70	May 27	71	65
5	59	48	30	71	52	29	78	65
6	69	53	May 1	63	60	30	71	67
7	75	72	2	69	65	31	73	67
8	80	71	3	75	69	June 1	76	70
9	76	62	4	76	67	3	77	66
10	62	52	7	85	67	4	75	70
11	56	50	8	74	64	5	70	67
12	59	50	9	72	61	6	68	66
13	60	54	10	64	60	8	75	70
14	66	58	11	66	64	10	74	65
15	78	62	12	73	60	11	79	75
16	72	56	13	74	63	12	74	68
17	65	55	14	75	66	13	77	70
18	75	59	15	71	70	14	75	70
19	83	75	16	79	69	15	78	74
20	82	61	17	72	65	16	79	70
21	65	57	18	66	60	17	80	70
22	67	61	20	68	62	18	74	70
23	78	65	21	67	58	19	73	66
24	63	53	22	61	57	20	72	68
25	78	51	23	69	59	23	85	72
26	60	58	24	79	71	25	92	83
27	73	54	26	78	64	26	89	81
28	70	59						

dition of, for example, the *A I* cultures at the end of the first fifteen days, with the *A III* cultures at the end of the same length of time without taking the temperature into account. However, it is fair to compare the relative time of appearance of the various organisms in *A I* and the relative time of appearance of the various organisms in *A III*; but even here an error is undoubtedly present, though, it is believed, it is not sufficiently marked to appreciably influence the general results though minor variations which occurred in particular infusions may well be due to it. This error arises from the fact that the different species of organisms in the infusions undoubtedly have their own optimum temperature for development and consequently it may be supposed that a particular form, which has a comparatively high optimum temperature, may reach its maximum later than another with a slightly lower optimum temperature, in the cultures existing during the early part of April when the general average temperature was lower, while it may attain its maximum earlier than the latter in the cultures which reached a corresponding stage of their development when the temperature was generally higher.

As already stated, the first intention was to follow the entire fauna and flora which developed in the infusions, but this involved more labor than could be performed accurately by one observer. Consequently although a record was kept of all the animals and plants which actually were observed, these data will not be presented because I am not satisfied that they are sufficiently accurate or comprehensive. One who has not attempted to follow in detail a series of cultures, started in the manner described, has not, I think, an adequate realization of the wealth of forms which will develop. Some of the forms appear and disappear with such marvellous rapidity that if they are not immediately identified, in many cases it is impossible to do it later. Therefore, I repeat that the description which follows simply affords the data collected in regard to certain well-known genera and groups of Protozoa, which appeared in sufficient numbers, in a large majority of the infusions, to render their study of value in attempting to reach some general conclusions as to their sequence in such infusions under the conditions of the experiment. It is believed that the concentration of attention on these few forms is prefer-

able to a wider consideration of many transient species which appear apparently at random, for, if it is possible to reach any conclusions of value from the study of these few dominant forms, it may open the way for an explanation of the seemingly fortuitous distribution of the remaining species.

A tabulation of the fauna of the infusions showed that the first analysis of the results should consider the following groups and genera of Protozoa: Monads, Colpoda, Oxytricha and various closely related hypotrichous forms, Paramaecium, Vorticella, and Amoeba, because all these organisms were present in practically every infusion. The term 'monads' is used in a broad sense to include several different genera and a multitude of species of small flagellate Protozoa usually classified under the generic names *Oikomonas*, *Monas*, *Bodo*, etc. Colpoda cucullus is the most common member of the genus Colpoda which has appeared in the infusions. Occasionally the form of the organism has not agreed exactly with the specific description usually given, and it may well be that some of these organisms properly rank as other species of the genus, but as this could be determined only by following out the life history of the animals, it was necessary to assign the forms merely to the genus. In a number of cases species of Colpidium was found intermixed with the Colpoda. Colpoda and Colpidium are apparently adapted to practically identical conditions of the infusions and consequently it matters little which form is chosen for study. Since Colpoda has usually appeared in greater abundance than Colpidium, it has been selected, as the representative of this type of ciliate, for detailed study in this work. Among the hypotrichous ciliates which appeared, Oxytricha was probably the most common, but closely associated with this genus was *Stylonychia*, *Urostyla*, *Gastrostyla*, etc., and therefore the various species of these genera were considered as a unit and are designated in this work as 'Hypotrichida.' Also several members of the Vorticellidae appeared, nearly all of the genus *Vorticella*. The term 'Vorticella' accordingly is used to include all true members of this genus regardless of species. The same is true of the term 'Amoeba' as here employed, this name being used to include such forms as *Amoeba guttula*, *radiosa*, etc., as well as typical *Amoeba proteus*. 'Paramaecium' is applied to two species,

aurelia and caudatum, indiscriminately. It is apparent, then, that no attempt has been made to identify the various species, as this would necessitate a large amount of labor entirely incommensurate with the value of the information gained for the problem in hand. All of the forms included together are adapted to the same general environment (as the results which follow show), and therefore it is logical to consider them together as a unit without regard to the taxonomic variations of the individual moieties of which it is composed.

B. GENERAL OBSERVATIONS ON THE COURSE OF DEVELOPMENT  
OF HAY INFUSIONS

In infusions (A) made with boiled hay, which is allowed to remain in the jar, most of the hay sinks quickly to the bottom and remains there. In the cultures (C) made with unboiled hay most of the material floats near the surface for four or five days and then begins to sink gradually to the bottom. It is usually all at the bottom within two weeks. When the hay is allowed to remain in the infusion (A, C) this slowly disintegrates and is reduced to a more or less amorphous mass by the end of the second month. The rapidity of these changes, however, varies considerably with the temperature to which the cultures are subjected.

When hay and water are combined the liquid rapidly becomes straw colored, and within the first few days bubbles of gas appear entangled amongst the hay at the bottom, and these rise by degrees to the surface. At comparatively high initial temperatures the gas will frequently disturb the hay and sometimes raise it to the surface. Peters' observations show that this gas is chiefly  $\text{CO}_2$ . By the third or fourth day the color of the culture liquid appears darker and this becomes increasingly pronounced until finally the liquid is of a dark brownish color. One familiar with infusions can, of course, readily tell the approximate age of a culture by its color. Fine's studies on these infusions show that the light and yellowish shades of color are due to relatively high acidity; the darker and brownish shades to relatively low acidity.<sup>10</sup>

<sup>10</sup> Fine. Loc. cit.

When the infusions are first made up, the liquid, though colored, is transparent, but within forty-eight hours it becomes markedly turbid due to the development of countless bacteria. The bacteria at this time are equally distributed throughout the medium but on the third day a 'zoogloea' begins to be established and gradually increases in amount until it finally falls to the bottom and another is formed. In some cases, however, the 'zoogloea,' after reaching its maximum thickness, at approximately the end of thirty days, gradually thins out and practically disappears *in situ*. These variations in the transformation of the 'zoogloea' introduce a complicating factor in the study of the protozoan life of infusions, because in the cases in which it falls to the bottom, it changes the center of population of certain types quite suddenly, and thus causes a redistribution of some forms. The bacteria, then, at first are equally distributed throughout the fluid, then the largest number is at the bottom and top, while in the center of the volume of liquid there are comparatively few. The hay and smaller amount of oxygen at the bottom, and the more abundant supply of oxygen at the top, offer attractions for different forms with the result that apparently approximately the same number are to be found in each region. After the 'zoogloea' has fallen or disappeared the center of bacterial life is again at the bottom amongst the remnants of the disintegrating hay.

As soon as the bacteria have become numerous, and their action on the hay has put a certain amount of it in a form available for animal life, then occurs the great growth of Protozoa, comprising saprophytic, herbivorous, carnivorous and omnivorous forms, and this phase of the life of the infusions we shall consider in detail.

After the period of greatest protozoan fauna has passed, rotifers become numerous, and as the diatoms, desmids, and filamentous cyanophyceae and chlorophyceae flourish, under proper conditions of illumination, several species of *Anguillula*, copepods, etc., are more or less abundant. This condition of the fauna and flora merges imperceptibly into what may be called a condition of nearly stable equilibrium, in which green plants and animals, under



optimum conditions of light and temperature, are so adjusted that for a considerable period a practically self-supporting and self-sufficient microcosm exists—but with the balance of nature established neither the Protozoa nor bacteria can ever again attain their maximum abundance.

#### C. THE *A*, *B* AND *C* GROUPS OF INFUSIONS

All three types of infusions (*A*, *B*, *C*) which were made up gave the same general cycle of events, but the *A* and *C* series were slightly slower in development (as one would expect from the presence of the hay) than the *B* series. The cycle of the *C* series was essentially the same as that of the *A* series except that it progressed somewhat more slowly until the hay became thoroughly soaked. A practical disadvantage of the *C* series is presented by the fact that the unboiled hay, containing considerable air, has a tendency to float and so changes somewhat the distribution of the organisms until it sinks to the bottom at about the end of two weeks. This nuisance may be avoided by weighting the hay with glass. So far as length of cycle is concerned, however, both the *A* and the *C* series offered equal advantages for study, but the cycle of the *B* series (without hay) being considerably shorter, the sequence of the different types of organisms was more rapid, the number of organisms present was much smaller, and stable equilibrium of the infusions was attained sooner (cf. figs. 5, 6, 7). However, since the richness of the animal life was seriously decreased, this series did not prove to be the best for study, and accordingly such a method of making up cultures is not recommended for investigations of this character. Nevertheless, the results derived from all three types of cultures will be given here.

The data from each of the twenty-six cultures have been recorded (as already described), then these data from each culture of each set of experiments of the *A*, *B*, and *C* series, started at the same time, have been averaged together. Therefore, in discussing these data, I shall refer (unless it is specifically noted to the contrary) to the *average* number of organisms, time of appearance, etc., in infusions comprising each group as follows:

A-1, A-2, A-3, A-4, A-5, A-6,	averaged and designated <i>A I</i>
A-21, A-22	averaged and designated <i>A II</i>
A-31, A-32	averaged and designated <i>A III</i>
A-41, A-42	averaged and designated <i>A IV</i>
B-1, B-2	averaged and designated <i>B I</i>
B-21, B-22	averaged and designated <i>B II</i>
B-31, B-32	averaged and designated <i>B III</i>
B-41, B-42	averaged and designated <i>B IV</i>
C-1, C-2, C-3	averaged and designated <i>C I</i>
C-31	designated <i>C III</i>
C-41, C-42	averaged and designated <i>C IV</i>

This method of treating the data was decided upon because it gives, it is believed, the fairest picture of the protozoan sequence in the infusions. As a matter of fact the individual infusions of the respective groups presented comparatively unimportant variations—except in certain cases which are mentioned. Three of the six infusions composing group *A I* were discontinued at the end of the first month because the variations between the individual infusions was not sufficient to warrant the study of so many. For a record of the surface sequence of a single infusion, reference should be made to *C III* (fig. 10). For the data of a single form (*Paramaecium*) at the bottom of two infusions comprising a single group, see fig. 12.

\*  
D. TIME OF APPEARANCE, MAXIMUM NUMBER AND DISAPPEARANCE  
OF REPRESENTATIVE PROTOZOAN FORMS AT THE SURFACE OF  
THE INFUSIONS

1. *Monad*

*A I group.* Monads were the first animals to appear in considerable numbers and their maximum was attained on the 7th day when there were about 5200 per cc. Their decline was equally rapid and by the 20th day of the life of the infusions none were observed in the samples studied.

*A II group.* These forms were the first to appear, reach their maximum of 2000 per cc. on the 4th day, and minimum on the 8th day.

*A III group.* Monads were practically absent from the two cultures of this group, and this is the only instance in which they did not appear in numbers sufficient to be considered. There were, perhaps, 100 per cc. at several counts. This dearth is accounted for, I think, by an exceptionally heavy growth of Colpoda, which occurred in this group very early (cf. table 2 and fig. 3).

*A IV group.* The monads appeared on the 2nd day, attained their maximum of 1000 per cc. on the 4th day and reached their minimum on the 6th day.

*B I group.* These forms appeared on the 2nd day, attained a maximum of 4200 per cc. on the 8th day, declined to 500 per cc. on the following day and then gradually became less and less until by the 23rd day their number was negligible. On the 36th day, however, they reappeared, attained the number of about 2000 per cc. on the 40th day, and reached extinction on the 60th day.

*B II group.* On the 4th day there were 1200 monads per cc., and on the 8th day they had entirely disappeared.

*B III group.* In this group the monads attained a maximum of 5000 per cc. by the 9th day, and by the 12th day there were none remaining.

*B IV group.* A maximum of 1400 per cc. was reached on the 3rd day of the life of the cultures, and then a rapid decline resulted in extinction by the end of the first week.

*C I group.* In these three cultures the average maximum number of monads, nearly 8000 per cc., occurred on the 15th day, and was followed by an abrupt decline ending with their disappearance on the 20th day. The maximum of Colpoda occurred on the same day as that of the Monads.

*C III group.* In this group, represented by a single infusion (C-31), the monads attained a maximum of over 8000 per cc. on the 8th day, declined rapidly to 2500 per cc. on the 13th day, and reached a minimum of practically zero on the 24th day.

*C IV group.* Here the monads rose to the number of 5000 per cc. on the 17th day, declined to about 2500 per cc. on the 21st day, then rose to their maximum of 7600 on the 27th day, and by the 32nd day had reached a minimum.

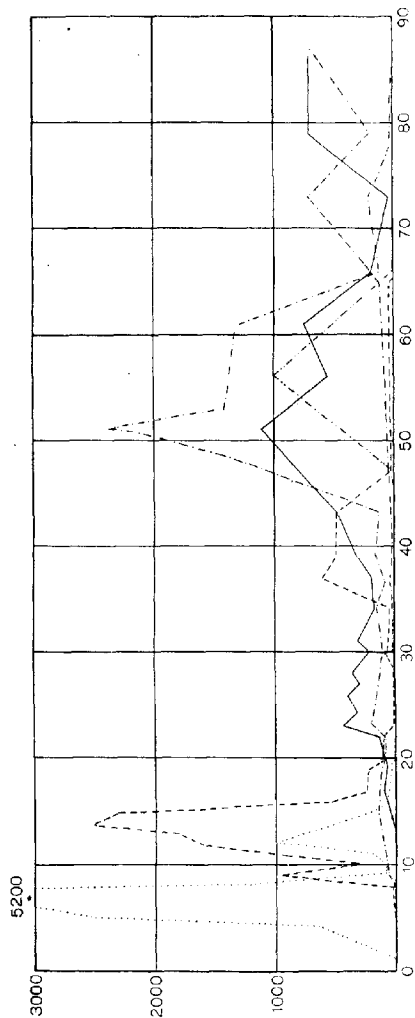


Fig. 1 *A I* group. Ordinates indicate the number of organisms per cc. at the surface. On the abscissa is plotted the number of days of the existence of the infusions. Monad = .....; Colpoda = - · - · -; Hypotrichida = - - - -; Paramecium = - - - -; Vorticella = ———; Amoeba = — · — · —. (For details of methods cf. p. 225.)

From the study of the monads in all the cultures it is clear that in every instance they were the first type of protozoon to appear and the first to reach a maximum. This is undoubtedly to be explained by the fact that these forms, combining holozoic and saprozoic methods of nutrition, are able to feed on the bacteria which are developing so rapidly at this period, and also to absorb various substances entering into solution from the hay. The monads under consideration are also the first forms to decline and practically disappear, and this is probably due, in part, to the rapid decrease in numbers of the bacteria brought about by the monads themselves and by the rising generations of Colpoda.

## 2. Colpoda

*A I group.* Colpoda was the second protozoon to appear in considerable numbers and its maximum was attained on the 14th day when there were about 2500 per cc. Its decline was equally rapid and by the 25th day very few active individuals were seen. Beginning at about the 30th day, however, more were observed and on the 37th day there were about 600 per cc. This second rise in numbers was followed by a more gradual decline which ended in the extinction of this form by the 66th day of the life of the infusions.

*A II group.* This form was the second to appear and very slowly attained its maximum of 1000 per cc., which took place on the 27th day, then it fell in number to about 200 per cc., rose again to about 500 per cc. on the 44th day, and then became extinct on the 49th day.

*A III group.* Colpoda was the second protozoon to appear in considerable numbers in these infusions, the cycle of the monads being apparently aborted. Colpoda arose abruptly to the great number of 15,000 per cc. on the 10th day, fell to about 11,000 per cc. on the following day, and by the 15th day very few active forms were observed. However, almost immediately it had another period of reproductive activity which brought up the number to about 4000 per cc. on the 29th day. After this second high point it decreased in number, but persisted until the 68th day

of the infusion's life. The growth of Colpoda in this group of infusions is remarkable for its abundance and persistence, for during the greater part of the life of the infusion, Colpoda was the form which dominated.

*A IV group.* Colpoda was the second form to attain its maximum, which occurred on the 13th day with 2500 per cc. present. This number persisted to the 17th day, and then a very quick decline ended in the extinction of the form four days later.

*B I group.* Colpoda was the third to attain its maximum, being preceded by the monads and the hypotrichida. Its maximum occurred on the 14th day and this was followed by a slow decline resulting in the disappearance of Colpoda on the 30th day.

*B II group.* Colpoda attained its maximum abundance on the 6th day, then rapidly proceeded to its extinction on the 15th day. The notably small development of Colpoda in this group of infusions is paralleled by that of all the other organisms in *B II*.

*B III group.* In this group of infusions Colpoda rose rapidly to a maximum of 8000 per cc. on the 18th day, and then fell even more rapidly to extinction on the 29th day. In this series of infusions Colpoda was again the dominant form, greatly outnumbering the hypotrichida and paramaecia whose small maxima occurred before its own.

*B IV group.* The appearance of Colpoda occurred relatively late, none being observed until the 6th day, and its maximum growth occurred on the 12th day, and its extinction on the 16th day. In this series it was the fourth form in point of time to reach its greatest abundance.

*C I group.* In these three cultures the average maximum number of Colpoda, 4500 per cc., occurred on the 15th day, after a rapid rise from the 7th day. Then there was an equally sudden decline to about 40 per cc. by the 22nd day, and this number gradually decreased until it became negligible at the 46th day.

*C III group.* Again in this culture the growth of Colpoda overshadowed that of all the other forms. Appearing on the 4th day it gradually increased until a maximum of about 15000 per cc. was attained on the 33rd day. This was sustained for four days and then a remarkable decrease brought it down to about

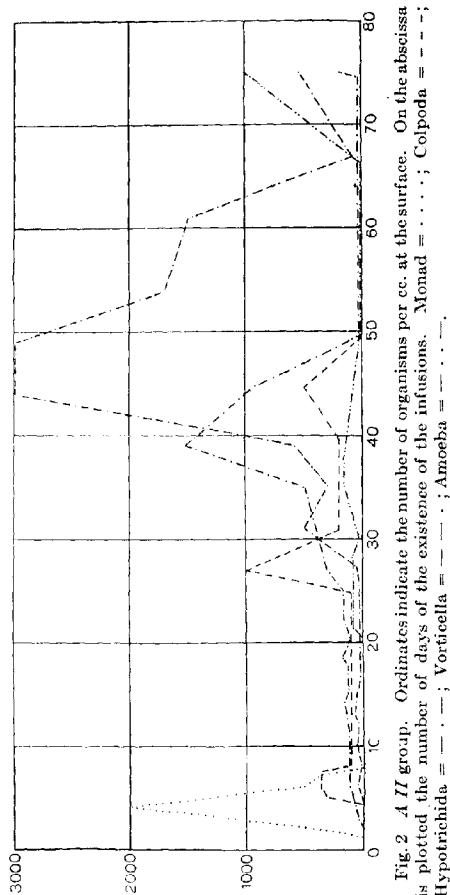


Fig. 2 *A II* group. Ordinates indicate the number of organisms per cc. at the surface. On the abscissa is plotted the number of days of the existence of the infusions. Monad = ....; Colpoda = ---; Hypotrachida = ---; Vorticella = ---; Amoeba = ---.

600 per cc. on the 43rd day, and 60 per cc. on the 49th day. Approximately this number persisted until the end of the observations on the 76th day.

*C IV group.* Colpoda developed in greater abundance in this group than in any other, attaining, comparatively gradually, a maximum of 25,000 per cc. on the 32nd day, falling to 15,000 on the 37th day, and to 100 per cc. by the 44th day, and practically reaching extinction by the 56th day.

An analysis of the above data in regard to Colpoda shows that this form is adjusted to those surface conditions of infusions which exist when the monads have about run their course.

### 3. *Hypotrichida*

*A I group.* Representatives of the hypotrichida were the third to appear in considerable number and their maximum of about 2400 per cc. was attained on the 51st day after a long gradual rise. Their decrease in number was somewhat more abrupt, resulting in their extinction by the 85th day of the life of the infusions.

*A II group.* These forms were the second to appear and the third to reach a maximum growth. This was attained on the 39th day after a long gradual increase. A rapid decline reduced them to about 40 per cc. on the 49th day, and they persisted in this number to the 74th day. There was a slight increase in number on the 75th day, at which time the observations were discontinued.

*A III group.* A few hypotrichida appeared on the 6th day, gradually increased until there were about 400 per cc. on the 15th and 16th days, then declined to the 24th day. Their maximum of 700 per cc. was reached on the 29th day, after which they declined until, on the 43rd, they became extinct.

*A IV group.* The maximum consisting of 1600 per cc. followed those of the monads and Colpoda and occurred on the 26th day. A continuous decline brought the number down to 100 per cc. by the 43rd day; and this number persisted to the 50th day,



after which there was a slight increase up to the end of the observations on the 57th day.

*B I group.* These forms attained a maximum of 560 per cc. on the 11th day, and then very gradually declined until they became practically extinct on the 55th day.

*B II group.* In this group the maximum of the hypotrichida was reached on the 11th day, and extinction on the 29th day.

*B III group.* The hypotrichida were practically negligible as the maximum number which occurred on the 10th day was less than 40 per cc., and the animals disappeared completely by the 15th day.

*B IV group.* Here these forms reached their greatest abundance, 250 per cc., on the 7th day, declined to about 20 per cc. by the 11th day, gradually rose to 150 per cc. on the 26th day, and disappeared by the 35th day.

*C I group.* In these three cultures the average maximum of about 360 per cc. occurred on the 19th day, and was followed by a continuous decline until the 49th day, when about 40 per cc. were seen. From this time on slight fluctuations in numbers occurred, and at the last observation on the 86th day, about 40 per cc. were still to be seen.

*C III group.* In this group, represented by a single infusion, these forms first appeared on the 20th day, reached their greatest abundance, 300 per cc., on the 24th day, then declined to about 40 per cc. on the 28th day, and persisted in approximately this number to the end of the observations.

*C IV group.* Here the hypotrichous forms reached a maximum of 500 per cc. on the 32nd day, declined to about 40 per cc. on the 37th day, rose to 400 per cc. on the 44th day, declined again to about 20 per cc. on the 51st day, and remained at about this number to the last observation on the 56th day.

From the study of the hypotrichous ciliates which appeared in all the cultures, it is clear that these forms are adjusted to conditions at the surface of the infusions which are present, in most cases, after the monads have nearly disappeared and Colpoda has passed its period of greatest abundance.

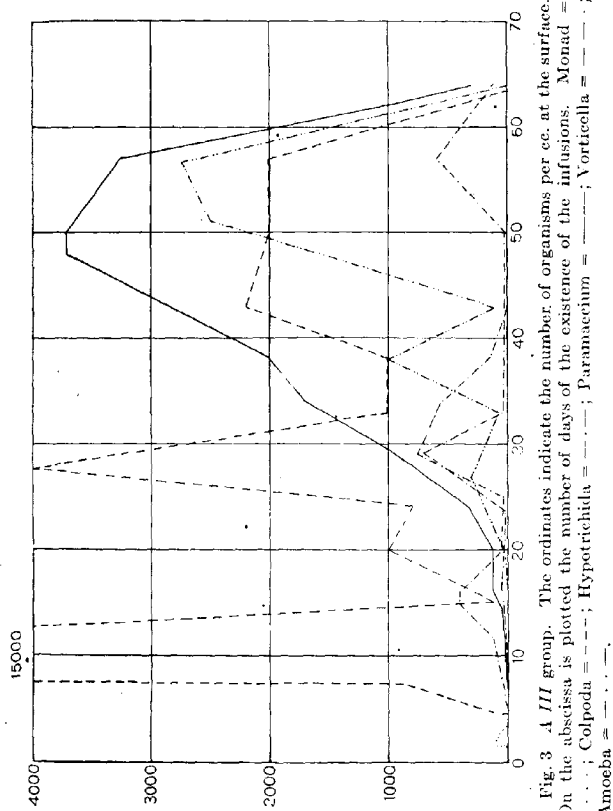


Fig. 3. A III group. The ordinates indicate the number of organisms per cc. at the surface. On the abscissa is plotted the number of days of the existence of the infusions. Monad = .....; Colpoda = - - - -; Hypotrichida = - - - -; Paramacrium = - - - -; Vorticella = - - - -; Amoeba = - - - -.

#### 4. *Paramaecium*

*A I group.* *Paramaecium* appeared in considerable numbers about the 14th day and after various fluctuations in numbers reached a maximum of 1100 per cc. on the 51st day. This was succeeded by a general decline in numbers to the 73rd day when only about 40 per cc. were present; but after this their number increased to about 700 per cc. on the 79th day and continued so to the end of the observations on the 86th day.

*A II group.* The data for the paramaecia of these two cultures will be considered later, because the presence of *Didinium* so altered the paramaecium cycle that it cannot be fairly compared with that in the other cultures.

*A III group.* *Paramaecium* made its appearance here on the 7th day and gradually increased to its greatest abundance on the 48th to 50th days, at which time about 3700 individuals per cc. were present. After this the number rapidly fell to 300 per cc. on the 64th day when the last count was made.

*A IV group.* This organism appeared on the 4th day, attained the number of 2500 per cc. on the 31st day, and this maximum continued for fourteen days after which there was an abrupt decline to about 50 per cc. on the 57th day, when observations were suspended.

*B I group.* *Paramaecium* appeared on the 10th day, attained a low maximum of less than 100 per cc. on the 20th day and then very gradually reached extinction by the 65th day.

*B II group.* This form was present in small numbers practically from the start (3rd day) and reached a small maximum of about 160 per cc. on the 7th day, from which time they gradually decreased until they became extinct on the 24th day. This culture also was influenced by the activities of *Didinium*, and this will be discussed later.

*B III group.* *Paramaecium* appeared on the 11th day, reached its maximum on the 17th day when 160 per cc. were counted. From this time to the 65th day, when the last count was taken, it continued to persist in varying numbers under 100 per cc.

*B IV group.* The cycle of *Paramecium* will not be considered at this time as it was so altered by *Didinium* that it is not at all comparable with that of the other cultures.

*C I group.* *Paramecium* appeared on the 21st day, gradually multiplied until 600 per cc. were counted on the 40th day, then increased rapidly to 2500 per cc. on the 45th day, fell to 1200 per cc. on the 48th day, and to 200 per cc. on the 61st day. From this time there was a gradual decline to the last count on the 86th day, when about 80 per cc. were present.

*C III group.* In this group, comprising but a single infusion, *paramecia* appeared on the 23rd day, increased to about 440 per cc. on the following day and fell in number until only about 10 per cc. were observed on the 34th day. From this point they

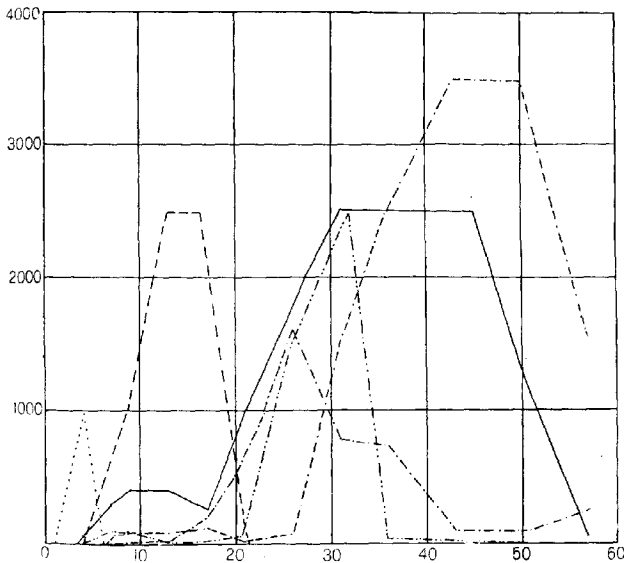


Fig. 4 *A IV group.* The ordinates indicate the number of organisms per cc. at the surface. On the abscissa is plotted the number of days of the existence of the infusions. Monads = .....; Colpoda = ----; Hypotrichida = - · - ·; Paramecium = ———; Vorticella = — · — ·; Amoeba = - - - -.

rose to a maximum of about 500 per cc. on the 37th to the 42nd day after which there was a general decline to 200 per cc. at the last observation on the 64th day.

*C IV group.* This organism appeared on the 31st day, attained its greatest abundance on the 44th day, with about 600 per cc. present, and then declined to 200 per cc. on the 65th day, when the final examination was made.

The results given above, when compared with those of the hypotrichida, show that *Paramecium* usually attains its maximum numbers at the surface when the hypotrichous ciliates have passed their period of greatest abundance.

#### 5. *Vorticella*

*A I group.* *Vorticella* made its appearance on the 30th day and increased gradually in numbers until the 65th day when about 100 per cc. were seen. Then it rose rapidly to a maximum of 700 per cc. on the 73rd day, declined equally rapidly to 200 per cc. by the 79th day, and then rose again to nearly 700 per cc. on the 86th day when the last observation was made.

*A II group.* This form appeared on the 5th day and persisted in small numbers to the 27th day. From this point it rose to 3000 per cc. on the 44th to the 49th day, and then quite rapidly declined to about 80 per cc. on the 67th day. Another rise to about 500 per cc. occurred on the 75th day when the culture was discontinued.

*A III group.* *Vorticella* appeared on the 8th day and continued in almost negligible numbers until the 25th day when it began to multiply rapidly until the maximum of 700 per cc. occurred on the 29th day. It then decreased to less than 20 per cc. by the 33rd day and remained in about this number to the 50th day, after which it again increased to 600 per cc. on the 57th day and declined to about 120 per cc. on the 64th day, when the last count was taken.

*A IV group.* This form made its appearance on the 7th day and persisted in comparatively small numbers until the 26th day,

when it began a period of great abundance, reaching a maximum of 3500 per cc. from the 43rd to the 50th day. It then declined to about 1600 per cc. on the 57th day when the last count was taken. *Vorticella* attained a higher maximum than any other protozoon in this group of infusions.

*B I group.* *Vorticella* appeared on the 18th day, rose to about 200 per cc. on the 21st day and fell to 20 per cc. on the 26th day. It continued in about this abundance up to the 34th day when it multiplied rapidly and produced a maximum of about 1000 per cc. by the 37th day. This was followed by a rapid decline for a few days and then a slow decline to the 67th day when the form became extinct.

*B II group.* *Vorticella* appeared on the 5th day and fluctuated in numbers under 200 per cc. until the 45th day when the maximum of 300 per cc. occurred. This was followed by a rapid decline resulting in extinction by the 50th day.

*B III group.* This genus appeared on the 16th day and persisted in very small numbers, reaching its maximum of 120 per cc. on the 59th day. It had decreased somewhat by the 64th day when the last observation was made.

*B IV group.* In this group *Vorticella* appeared on the 2nd day and persisted in numbers less than 200 per cc. until the 11th day, then arose abruptly to 3500 per cc. on the 16th day, and fell almost equally abruptly to about 20 per cc. by the 26th day. It persisted in approximately this number to the last count on the 36th day.

*C I group.* In this group of three infusions, the curve for *Vorticella* shows a peculiar series of fluctuations. The form appeared on the 44th day, rose to 500 per cc. by the 57th day, fell to 20 per cc. by the 67th day, rose again to practically 500 per cc. by the 74th day, fell again to about 20 per cc. by the 81st day and then had still another rise which brought the organism to its maximum on the 86th day, when the last observation was taken.

*C III group.* *Vorticella* appeared and attained its maximum of 100 per cc. on the 38th day in this infusion. From this time it very gradually decreased in numbers until the 65th day when the final count was made which showed about 50 per cc.

*C IV group.* Again in this series *Vorticella* passed through a series of fluctuations, beginning on the 19th day, reaching a maximum on the 32nd day of approximately 500 per cc., falling to nearly zero on the 37th day, and thereafter increasing in number until there were about 240 per cc. present at the final count on the 56th day.

A study of the data presented above shows that *Vorticella* usually attains its greatest abundance later than *Paramaecium*.

#### 6. *Amoeba*

*A I group.* *Amoeba* was first seen on the 47th day, rose to a maximum of about 1000 per cc. by the 56th day and decreased until none were present on the 67th day.

*A II group.* This form appeared on the 21st day and fluctuated in numbers until the 50th day when it completely disappeared. It reappeared again on the 66th day and reached a maximum of about 1000 per cc. on the 75th day when the last count was made.

*A III group.* *Amoebae* appeared on the 17th day and after various fluctuations attained a maximum of 2700 per cc. on the 57th day. After this they declined rapidly and had disappeared by the 64th day when the last count was taken.

*A IV group.* These forms were first seen on the 5th day and continued to be present, though in practically negligible numbers, until the 21st day. Then they began to multiply rapidly and attained a maximum of 2500 per cc. on the 32nd day. A sudden decline brought the number down to about 40 per cc. on the 36th day and zero was reached by the 50th day.

*B I group.* *Amoebae* did not appear at all in one of the cultures of this group, and in the other a total of 22 *amoebae* were observed at different times from the 40th to the 87th day.

*B II group.* Starting on the 23rd day, this form reached a period of greatest abundance on the 45th day when about 100 per cc. were counted. Extinction occurred by the 55th day.

*B III group.* This form appeared on the 14th day and reached a maximum of 1000 per cc. on the 25th day. They completely disappeared by the 37th day.

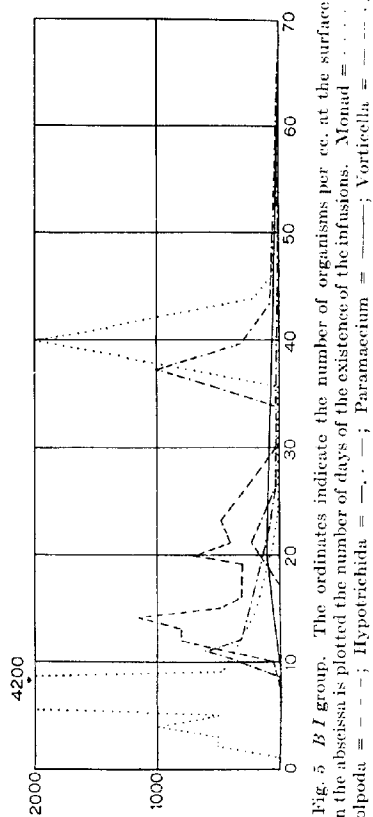


Fig. 5 *B I* group. The ordinates indicate the number of organisms per cc. at the surface. On the abscissa is plotted the number of days of the existence of the infusions. Monad = .....; Colpoda = - - - -; Hypotrichida = - - - -; Paramacium = - - - -; Vorticella = - . - .



*B IV group.* About 80 amoebae per cc. were observed on the 7th day, and from this they gradually decreased until the 18th day when the last individuals were seen.

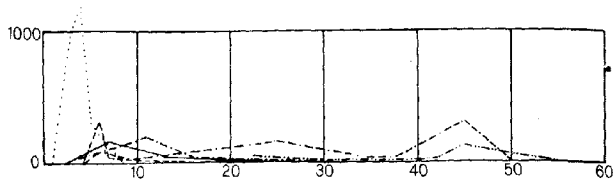


Fig. 6 *B II group.* The ordinates indicate the number of organisms per cc. at the surface. On the abscissa is plotted the number of days of the existence of the infusions. Monad = .....; Colpoda = - - - -; Hypotrichida = ~ - - -; Paramacium = - - - -; Vorticella = - - - -; Amoeba = - - - -.

*C I group.* Amoeba was first seen on the 46th day, reached its highest point one day later and maintained this maximum of nearly 300 per cc. for five days, after which a slow but steady decline brought it to extinction by the 86th day.

*C III group.* This form appeared on the 42nd day, reached its greatest abundance, 120 per cc., on the following day, and disappeared by the 53rd day.

*C IV group.* In this culture amoebae reached a greater development than in any of the others. They were first seen on the 6th day and reached 5000 per cc. from the 18th through the 22nd day. Then they declined quickly to about 500 per cc. on the 27th day, only to rise again almost as fast to their maximum of 5700 per cc. on the 38th day. From this point they fell to 200 per cc. by the 51st day, and at the last observation, on the 56th day, about 1000 per cc. were again present.

A careful analysis of the above data shows that amoebae attained their greatest development slightly later than *Paramacium* and earlier than *Vorticella* at the surface of the infusions.

## IV. SUMMARY OF SURFACE COUNTS

TABLE 2

		DAY OF APPEARANCE	DAY OF MAXIMUM	DAY OF DISAPPEARANCE
A I	Monads.....	2	7	20
	Colpoda.....	6	14	66
	Hypotrichida.....	9	51	85
	Paramecium.....	14	51	—*
	Vorticella.....	30	73	—*
A II	Amoeba.....	47	56	67
	Monads.....	2	4	8
	Colpoda.....	5	27	49
	Hypotrichida.....	3	39	—
	Paramecium.....	† 0	0	0
A III	Vorticella.....	5	44	—
	Amoeba.....	21	75	—
	Monads.....	2	2	3
	Colpoda.....	5	10	63
	Hypotrichida.....	6	29	43
A IV	Paramecium.....	7	48	—
	Vorticella.....	8	29	—
	Amoeba.....	17	57	64
	Monads.....	2	4	6
	Colpoda.....	5	13	21
B I	Hypotrichida.....	6	26	—
	Paramecium.....	4	31	—
	Vorticella.....	7	43	—
	Amoeba.....	5	32	50
	Monads.....	2	8	60
B I	Colpoda.....	8	14	30
	Hypotrichida.....	9	11	55
	Paramecium.....	10	20	65
	Vorticella.....	18	37	67
	Amoeba.....	0	0	0

\* A dash in this column indicates that the organism was still present when the last observation was made.

† Omitted because Didinium affected the sequence.

‡ Disturbed by Didinium.

TABLE 2—Continued

		DAY OF APPEARANCE	DAY OF MAXIMUM	DAY OF DISAPPEARANCE
B II	Monads.....	2	4	8
	Colpoda.....	5	6	15
	Hypotrichida.....	3	11	29
	Paramaecium.....	13	7	24
	Vorticella.....	5	45	50
	Amoeba.....	23	45	55
B III	Monads.....	5	9	12
	Colpoda.....	5	18	29
	Hypotrichida.....	8	10	15
	Paramaecium.....	11	17	—
	Vorticella.....	16	59	—
	Amoeba.....	14	25	37
B IV	Monads.....	2	3	7
	Colpoda.....	6	12	16
	Hypotrichida.....	3	7	35
	Paramaecium.....	10	0	0
	Vorticella.....	2	16	36
	Amoeba.....	7	7	18
C I	Monads.....	6	15	20
	Colpoda.....	8	15	46
	Hypotrichida.....	16	19	—
	Paramaecium.....	21	45	—
	Vorticella.....	44	86	—
	Amoeba.....	46	47	86
C III	Monads.....	3	8	24
	Colpoda.....	4	33	—
	Hypotrichida.....	20	24	—
	Paramaecium.....	23	37	—
	Vorticella.....	38	38	—
	Amoeba.....	42	43	53
C IV	Monads.....	2	27	32
	Colpoda.....	5	32	56
	Hypotrichida.....	18	32	—
	Paramaecium.....	31	44	—
	Vorticella.....	19	32	—
	Amoeba.....	6	38	—

TABLE 3<sup>11</sup>

	TIMES APPEARED FIRST	TIMES ATTAINED MAXIMUM FIRST	*TIMES DISAPPEARED FIRST
Monad.....	10	10	10
versus			
Colpoda.....	0	0	1 (B I)
Colpoda.....	8	6	8
versus			
Hypotrichida..	3 (A II, B II, B IV)	4 (B I, B III, B IV, C III)	2 (A III, B III)
Hypotrichida..	7	7	4
versus			
Paramaecium..	1 (A IV)	0	0
Paramaecium..	8	7	2
versus			
Vorticella.....	1 (C IV)	2 (A III, C IV)	0
Paramaecium..	7	7	0
versus			
Amoeba.....	1 (C IV)	1 (C IV)	6
Vorticella.....	7	4 (A II, A III, C III, C IV)	1 (B II)
versus			
Amoeba.....	3 (A IV, B III, C IV)	5	7

From the above tables the most frequent sequence for the entire series of infusions is found to be as follows:

APPEARANCE	MAXIMUM	DISAPPEARANCE
(1) Monad	(1) Monad	(1) Monad
(2) Colpoda	(2) Colpoda	(2) Colpoda
(3) Hypotrichida	(3) Hypotrichida	(3) Hypotrichida
(4) Paramaecium	(4) Paramaecium	(4) Amoeba
(5) Vorticella	(5) Amoeba <sup>12</sup>	(5) Paramaecium
(6) Amoeba	(6) Vorticella	(6) Vorticella

<sup>11</sup> Cases in which both of the organisms being compared attained the condition on the same day are not included. The fact that both the organisms frequently survived the period of observation (except in Series B) is responsible for the relatively few cases included in the third column.

<sup>12</sup> The figures for Amoeba versus Vorticella are so nearly the same that the variation is well within the error of the experiments.

A similar analysis of the data of the *A*, *B*, and *C* series of infusions separately shows the following sequence:

<i>A Series</i>	
APPEARANCE	MAXIMUM
(1) Monad	(1) Monad
(2) Colpoda	(2) Colpoda
(3) Hypotrichida	(3) Hypotrichida
(4) Paramecium	(4) Paramecium
(5) Vorticella	(5) { Vorticella
(6) Amoeba	Amoeba

<i>B Series</i>	
APPEARANCE	MAXIMUM
(1) Monad	(1) Monad
(2) { Colpoda	(2) { Colpoda
Hypotrichida	Hypotrichida
(3) Paramecium	(3) Paramecium
(4) Vorticella	(4) Amoeba
(5) Amoeba	(5) Vorticella

<i>C Series</i>	
APPEARANCE	MAXIMUM
(1) Monad	(1) Monad
(2) Colpoda	(2) Colpoda
(3) Hypotrichida	(3) Hypotrichida
(4) Paramecium	(4) Paramecium
(5) Vorticella	(5) Vorticella
(6) Amoeba	(6) Amoeba

#### V. PROTOZOAN FAUNA AT THE MIDDLE OF THE INFUSIONS

It is evident, from the observations on these infusions, that the protozoan fauna of the middle of the infusions is meager, compared with that of the top and bottom. Practically all the organisms which have been observed at either the top or bottom have been found in the middle counts; but either in such small numbers, or so irregularly, as to make a detailed tabulation of the records of little value. Therefore they are not presented here. Biologically, the middle of the infusion clearly offers a less favorable environment than either the top or the bottom, and is therefore tenanted chiefly by a free-swimming population brought there by an overcrowding at the top or bottom, and by forms emigrating from the top to the bottom as the cycle proceeds. Naturally

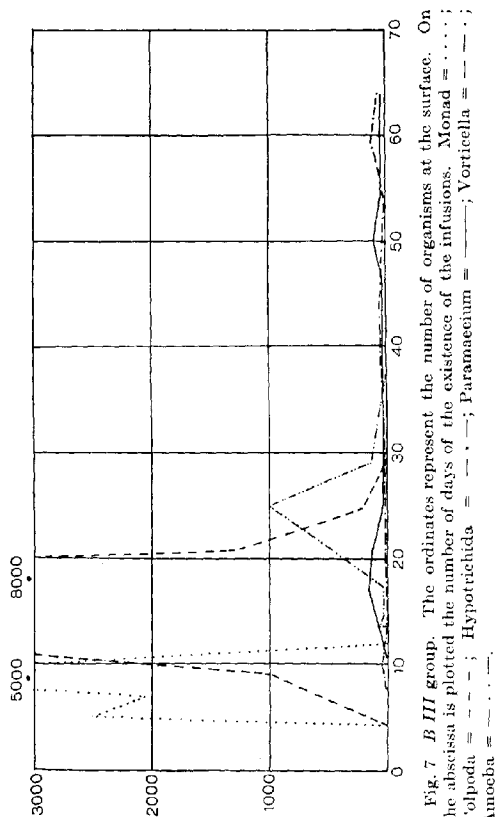


Fig. 7 *B III* group. The ordinates represent the number of organisms at the surface. On the abscissa is plotted the number of days of the existence of the infusions. Monad = .....; Colpoda = - - - -; Hypotrichida = - · - · -; Paramecium = - - - -; Vorticella = ·····; Amoeba = ———.

those protozoa, like *Paramecium*, which are strong swimmers are most frequently found in this region.

#### VI. PROTOZOAN FAUNA AT THE BOTTOM OF THE INFUSIONS

On account of the marked difference in the bottom fauna of the *A*, *B*, and *C* infusions, it is more convenient to consider each of these types of infusions separately.

##### 1. *A* Infusions

*Monad.* The types of monads recorded in the surface fauna were observed in inappreciable numbers at the bottom, so that it is evident that when these forms disappear from the surface their cycle is over. Certain other species of monads appeared irregularly in comparatively small numbers at the bottom, but it is unnecessary to recount them here.

*Colpoda.* In groups I and II Colpoda did not appear at all at the bottom. In group III comparatively few Colpoda (approximately one-twenty-fifth as many as at the top) appeared just during the top maximum. Group IV showed a maximum of 500 per cc., which coincided with the top maximum of 2500 per cc.

*Hypotrichida.* These forms occurred in negligible number in groups II, III and IV. In group I there was a small maximum of 60 per cc. on the 38th day.

*Paramecium.* Practically no paramaecia appeared at the bottom in any of the *A* cultures except *A*2, where, toward the end of the observations, one count of 300 per cc. was taken.

*Vorticella.* Vorticella were not observed in group I until near the end (76th day) when a maximum of 40 per cc. was attained. Group II, however, showed the largest number for the *A* series, with a maximum of 400 per cc. on the 72nd day, i.e., near the end of the observations. In groups III and IV Vorticella was not observed until nearly the end of the study when maxima of about 40 per cc. were reached.

*Amoeba.* In all the groups of infusions, amoebae were in greater abundance at the bottom than at the top. A maximum of 3000 per cc. occurred from the 55th to the 60th day in *A* I; a maximum of 6000 per cc. on the 76th day in *A* II; a maximum of 2000 per

cc. from the 36th to the 50th day in *A III*; and a maximum of 6500 per cc. on the 23rd day in *A IV*.

## 2. *B Infusions*

*Monad.* In groups *B I* and *B II* monads were practically absent. *B III* had none at the bottom during their presence at the top, but later a few were observed at the bottom from the 35th to the 55th day. In *B IV* monads appeared in numbers be-

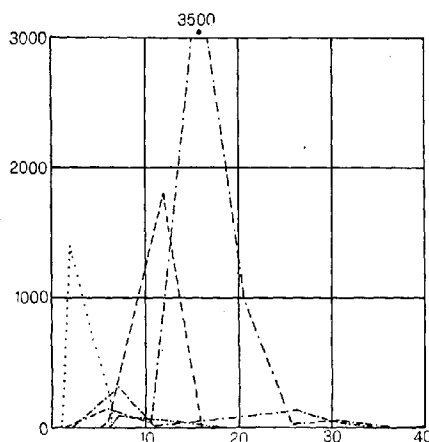


Fig. 8 *B IV* group. The ordinates represent the number of organisms at the surface. On the abscissa is plotted the number of days of the existence of the infusions. Monad = .....; Colpoda = ----; Hypotrichida = - · - ·; Vorticella = - - -; Amoeba = - · · ·.

tween 500 and 100 per cc. from the 20th to the 35th day. Their appearance was coincident with the descent of paramaecia. It will be recalled that few monads were observed at the surface of the infusions of this group.

*Colpoda.* Considerable diversity existed between the Colpoda in the *B* groups. In *B I* and *B III* they attained a temporary maximum of approximately 15000 per cc., just after their disappearance from the top (cf. fig. 13). In *B II* and *B IV* practically none were seen at the bottom.



*Hypotrichida.* These forms appeared in negligible numbers at the bottom in groups II and III, while in group I the largest bottom count was taken, i.e., 110 per cc. on the 33rd day (cf. fig. 14). Group IV also showed a relatively large bottom count as compared with the top count, having a maximum of 80 per cc. on the 24th day.

*Paramaecium.* *B I* showed a larger number of *Paramaecia* at the bottom than at the top. Conjugating specimens were seen at the bottom only (cf. figs 5 and 12). *B II* showed practically no paramaecia at the bottom, but this is explained by the fact that *Didinium* early exterminated them in this group. A heavy growth appeared in *B III* after conjugation was prevalent at the top (cf. fig. 13). *B IV* had very few paramaecia at the bottom until the 34th day and then there appeared about 500 per cc. All disappeared by the 45th day. Their appearance at the bottom was coincident with a decline at the top which was brought about by *Didinium*.

*Vorticella.* In group I this form attained a maximum of 175 per cc. on the 38th day, while in group II they reached a maximum of 240 on the 20th day. *Vorticella* appeared in group III in small numbers at both top and bottom, the bottom maximum being 60 per cc. on the 31st day. In group IV, however, the largest bottom count was recorded, i.e., 600 per cc. on the 28th day (cf. fig. 15).

*Amoeba.* There was a great difference in the amoeba fauna of *B1* and *B2* of group I, so that it is better to present these separately. *B1* had a maximum of 10000 per cc. on the 53rd day, while *B2* contained practically no amoebae at any time. The *B II* group showed a large growth which attained a maximum of 2500 per cc. on the 50th day and terminated on the 58th day. There was a relatively small maximum of 250 per cc. on the 37th day in *B III* and the amoebae had disappeared by the 45th day. A maximum of 2000 per cc. was in existence in *B IV* from the 35th to the 45th day, when the last regular observation was made. This culture, however, supplied countless *Amoeba proteus* for class use for two years thereafter.

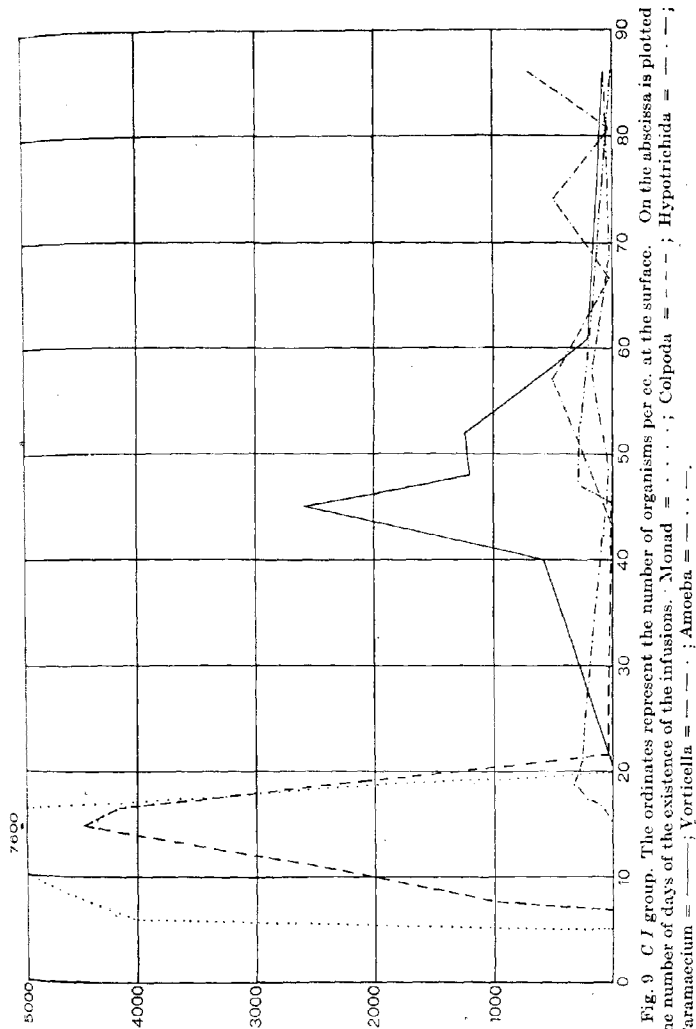


Fig. 9 C J group. The ordinates represent the number of organisms per cc. at the surface. On the abscissa is plotted the number of days of the existence of the infusions. Monad = .....; Colpoda = - - - -; Hypotrichida = - - - -; Paramaecium = ———; Vorticella = - - - -; Amoeba = - . - . - .

### 3. *C Infusions*

*Monad.* Monads appeared only in inappreciable numbers in all the groups of infusions. In group IV, however, a number of monad forms, other than those included in the top counts, appeared in considerable numbers for a time.

*Colpoda.* Practically no Colpoda were recorded for groups I and III. Group IV showed a brief maximum of 2500 per cc. which coincided with that of the top.

*Hypotrichida.* The hypotrichous fauna was practically zero.

*Paramaecium.* Paramaecia were not observed, except in group I where 100 per cc. were recorded for five days after a rapid decline at the top.

*Vorticella.* Practically no Vorticella appeared in the bottom counts.

*Amoeba.* In group I a few amoebae appeared on the 45th day and reached a maximum of 250 per cc. within the next five days, and then disappeared with equal rapidity. A heavy growth of 10000 tiny amoebae was attained in group III by the twenty-fifth day, and all were practically gone within ten days. In group IV a maximum of 10000 tiny amoeba was recorded on the 20th day and from this time the number gradually decreased until the 47th day when very few were observed. This decline was followed by a rapid rise to about 2000 per cc. on the 56th day when the last count was taken.

## VII. DISCUSSION AND CONCLUSIONS FROM THE OBSERVATIONS ON THE SEQUENCE OF THE SURFACE, MIDDLE AND BOTTOM FAUNA

### 1. *Surface fauna.*

These extended observations on the protozoa of typical laboratory infusions, made up by several different methods, clearly indicate a definite succession of certain representative forms at the surface of the water.<sup>13</sup>

<sup>13</sup> I am indebted to Mr. T. S. Painter, one of my students, who made for me a careful study of a number of similar infusions in the Yale Laboratory and also at his home in Salem, Virginia. His observations show an essentially comparable

The close agreement both of the sequence of appearance and of maximum numbers in all three series (*A*, *B*, *C*) is striking (cf. p. 244) and indicates that the sequence is not merely the result of factors incidental to the methods employed.

The data in regard to the time of disappearance is relatively meagre for the *A* and *C* series because many of the typical forms studied survived the period of the last observation. Consequently the sequence of time of disappearance is based chiefly on data from the *B* series, which, on account of the removal of the hay, passed through its cycle much more rapidly.

It is remarkably suggestive that the sequence (derived from the entire series of infusions) of all the forms at the time of appearance and at the time of maximum numbers and at the time of disappearance is *identical*, with the exception of *Amoeba*. The data indicate that the *Amoeba* cycle in the infusions is comparatively short since the position of *Amoeba* in the series advances progressively forward: it being last at the time of appearance, next to last (before *Vorticella*) at the time of maximum and third from last (before *Paramaecium* and *Vorticella*) at the time of disappearance. However, as has been already pointed out, the data is not sufficient to positively establish the relative position of *Amoeba* and *Vorticella* at the period of maximum numbers.

A study of the curves plotted from the surface counts of single infusions or groups of infusions reveals the fact that when once a great development is attained by a particular form, this maximum is seldom approached again. There are, however, some striking exceptions to this as, for example, *Colpoda* in group *A III* (cf. fig. 3) and the *Hypotrichida* in group *C IV* (cf. fig. 11). The curves further show that the major rise and fall in numbers are usually of about equal rapidity, though the final complete disappearance of an organism from the infusion may be long deferred. Careful searching in many of the *A* and *B* infusions

sequence of forms with the one here described. Among the monads, however, he found a large development of *Chilomonas*, while this form was relatively scarce in my infusions. Also, his *Amoeba* fauna was partially replaced by a considerable growth of *Arcella*. This latter result is interesting since it shows that somewhat closely related rhizopods fill substantially the same place in the economy of the infusions.

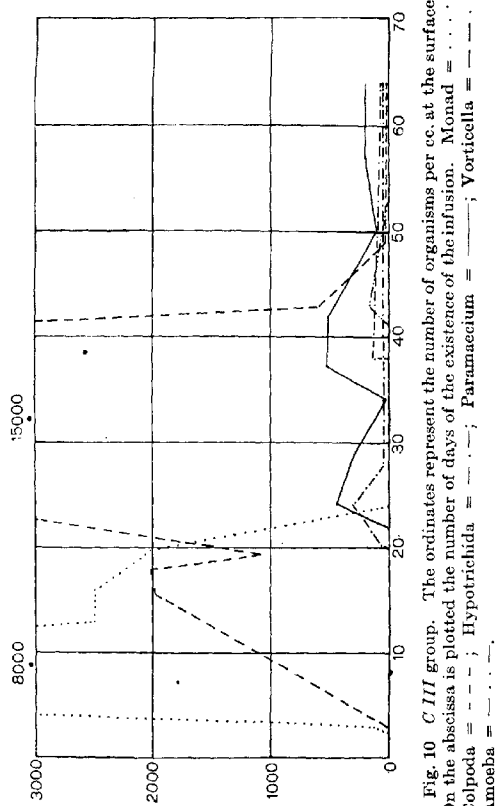


Fig. 10 *C III* group. The ordinates represent the number of organisms per cc. at the surface. On the abscissa is plotted the number of days of the existence of the infusion. Monad = - - - - -; Colpoda = - - - - -; Hypotrichida = - - - - -; Paramecium = - - - - -; Vorticella = - - - - -; Amoeba = - - - - -.

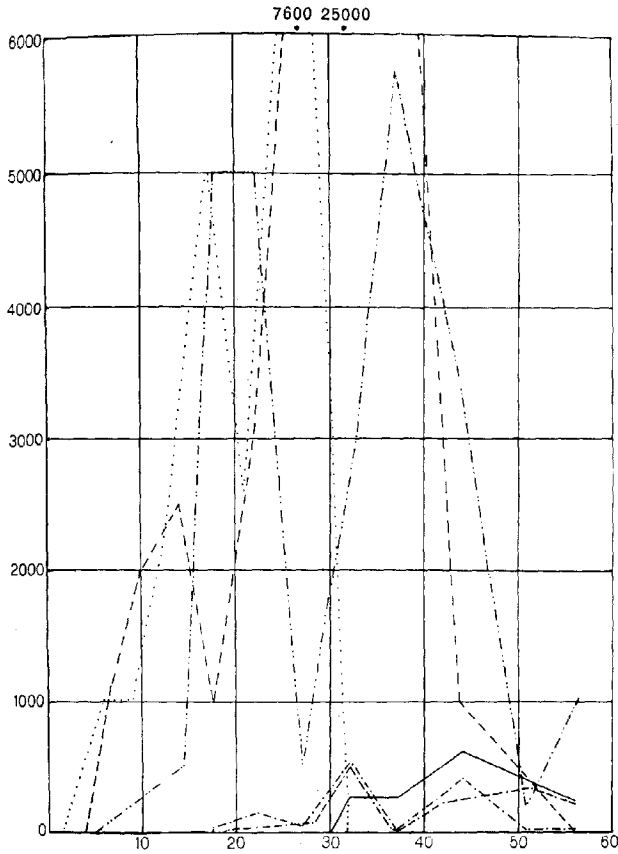


Fig. 11 *C IV* group. The ordinates represent the number of organisms per cc. at the surface. On the abscissa is plotted the number of days of the existence of the infusions. Monad = .....; Colpoda = - - - -; Hypotrichida = - . - . -; Paramaecium = ———; Vorticella = - - - - -; Amoeba = - - - - -.

after a lapse of nearly three years showed a few survivors of nearly all the chief forms, mostly at the bottom among the algae and débris.

### 2. *Middle fauna*

It is impossible to determine any definite sequence of forms for the *middle* of the infusions—this region being, as already pointed out, a more or less neutral territory which is encroached upon from time to time by organisms from the top and bottom as conditions in these regions vary.

### 3. *Bottom fauna*

The *bottom* fauna also has not exhibited a definite succession similar to that of the top. A study of the data already presented shows that the protozoan forms under consideration, with the exception of many amoebae, are essentially surface dwellers and seldom resort to the bottom except during or after a period of great development at the top. However, there is no invariable correlation between a fall in numbers at the top and a rise in numbers of the same species at the bottom, and it seems clear that, in the majority of cases, when a species declines in one region, most of the animals encyst or die. The latter is certainly true for *Paramecium* because many hundreds of passive and dying individuals, affording a feast for *Coleps*, may sometimes be seen among the débris at the bottom. Again, myriads of cysts of hypotrichous forms are frequently found at the bottom as the surface decline proceeds. Amoebae, among the protozoa under consideration, appear to give some evidence of migrating from the surface to the bottom which is their chief abode. The data on amoebae give the impression that some forms first appear in the infusions as amoeboid-flagellates which gradually increase in size and before long are unable to assume the flagellated phase. The pseudopodia of these are first of the guttula type but become more and more long and slender until many typical *radiosa* forms are present, and these in turn give place to typical large *A. proteus*. Only in certain infusions has it been possible to trace such a series, but

in these it has been quite striking, and in one of the later infusions I was able to predict correctly that declining amoeboid-flagellates would be replaced by typical amoebae. Such a cycle, of course, would not be remarkable in view of the results of some investigations on amoebae.<sup>14</sup> Although the data from these infusions by no means prove that the forms represented in this cycle are stages in the life history of a single species, nevertheless I lean toward the view that such will prove to be the case (cf. p. 211).

Taken as a whole, the study of the bottom fauna has proved to be less interesting than was anticipated, as I had expected to find

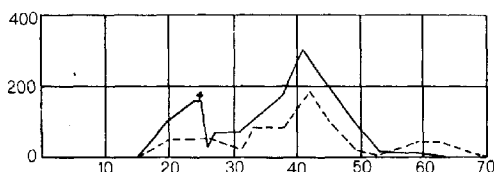


Fig. 12 Comparison of the *Paramaecia* fauna at the bottom of infusion B1 (—) and infusion B2 (---). x = point at which an epidemic of conjugation occurred in B1.

a much closer correlation between declines at the top and rises at the bottom, and *vice versa*. Apparently the bottom forms are largely independent of those at the surface, and the protozoan types under consideration, with the exception of the amoebae, are represented at the bottom by considerable numbers of active individuals chiefly when some sudden change, such as the falling of the zoogloea, brings them down, or by stragglers which manage to exist by avoiding the competition at the top. It is nearly always possible, by careful searching, to find at the bottom a few struggling individuals which have survived from an earlier prosperous surface population.

<sup>14</sup> For example, cf. Metcalf: *Studies upon Amoeba*. Jour. Exp. Zool., vol. 9, 1910.



#### 4. *Factors determining the sequence*

The problem becomes enormously complex when an attempt is made to decide upon the chief determining factors of the observed sequence of organisms at the surface of the infusions, and is entirely beyond our power of analysis from the data extant. Therefore, I believe, it is preferable at this time not to enter into an extended discussion of this question. I shall, however, briefly mention some points which seem to indicate suggestive lines for future study.

There is experimental evidences that, broadly speaking, the potential of division decreases from monads to paramaecia; that is, for example, paramaecia, under optimum conditions, divide less frequently than the majority of the hypotrichida, and similarly, the latter divide less rapidly than Colpoda. In regard to Vorticella and Amoeba, however, sufficient data are not at hand to make a definite statement.

With this in mind a series of experiments were made on the time of appearance of maximum numbers of Monads, Colpoda, Hypotrichida and Paramaecium in separate flasks of infusion which were seeded with a single individual of one species. The multiplication of the respective forms in the various flask cultures was observed, and the results showed remarkable agreement with the sequence of maximum numbers as determined for these same forms in the regular infusions. Consequently it appears that the number of specimens of any particular organism initially introduced into the large infusions, or the time of emergence of encysted forms has not had an important influence on the sequence of maximum numbers in these infusions as determined for the complete series. It may well, however, account for at least some of the individual variations in the sequence of appearance in numbers sufficient to be included in the samples studied, and of maximum numbers, which are apparent in particular groups of infusions. Again the interaction of the different forms would appear, at first glance, not to be a crucial factor in the sequence of maximum numbers since, in the experiments cited, the 'sequence' was duplicated, when only one species of organisms was

in each flask of infusion. This conclusion nevertheless, does not necessarily follow from the data, because all of the forms under consideration can flourish on a bacterial diet, which, of course, was supplied in each case. The interaction of the various forms clearly plays a part in the duration of the maximum and the rapidity of the decline. Experiments by the slide method of culture, which I have employed in my pedigree culture work, show that in culture medium which is the same from day to day practically the same 'sequence' of maximum numbers occurs and in this case it is apparent that chemical changes in the environment are not responsible for the results. Further, it is possible to carry all the forms under consideration for at least one hundred generations by this slide method, and this is sufficiently long to show that enough organisms can be produced in a medium which is chemically constant to supply, many times over, the number of organisms recorded at the maxima in the regular infusions. Consequently I think that these observations indicate that the relative potential of division of the four forms under discussion is adequate, under certain conditions at least, to establish the observed sequence of maximum numbers, and clearly suggest that it may be an important factor in large infusions.

The data from these infusions lead me to believe that the strictly biological factors are of greatest importance, and that it is necessary to look to somewhat subtle chemical changes in the medium for the important chemical factors in the environment. Fine's studies<sup>15</sup> on these infusions are in accord with this view and indicate that such general chemical changes in the environment as, for example, titratable acidity are not determining factors, at least for these particular species. My work on the excretion products of *Paramecium* shows,<sup>16</sup> however, that such substances have an inhibiting influence on the reproduction of this form, and it is quite probable that these products affect the sequence, maximum numbers, and decline of the various species. In fact Shelford, in his studies on the ecological succession of fish in ponds, believes that

<sup>15</sup> Cf. Fine: loc. cit.

<sup>16</sup> The effect of excretion products of *Paramecium* on its rate of reproduction. *Jour. Exp. Zool.*, vol. 10, no. 4, 1911.

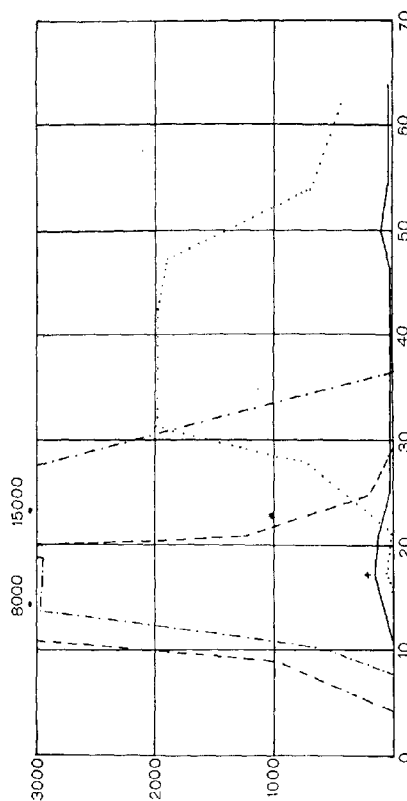


Fig. 13 *B III* group. Colpoda at the surface = — — — and at the bottom = — — —; Paramacium at the surface = — — — and at the bottom = — — —; x = point at which an epidemic of conjugation occurred at the surface.

his data show that the succession of those forms is not determined by the kind of available food but to an unused increment of decomposition and excretory materials which, in the last analysis, affects breeding.<sup>17</sup>

### 5. Decline in numbers

Closely involved with the problem of the sequence of appearance and maximum of the various forms is that of their more or less rapid decline in numbers. Here again the accumulated data do little more than establish the fact. The decline of the monads is quite clearly due, in part at least, to the evident variation in the amount of food in solution and to the rising hosts of Colpoda. The decline of Colpoda may be similarly ascribed to the dominance of the hypotrichida. Most of the hypotrichous forms were literally filled with ingested Colpoda which formed their staple diet. The relations of Paramecium and Vorticella to their predecessors, successors and to each other is not so apparent, but their abundance may well be influenced by a succession of the bacterial flora, for example, which unfortunately could not be followed in these studies, as well as to the host of other protozoan species. The competition between the various forms is so keen and the cycle is so rapid that even daily observations are, at times, insufficient to reveal the kaleidoscopic changes. Now and then, however, some prominent case of competition, such as that between Paramecium and Didinium, is forced upon the attention and the reason for the extinction of one form is clear. Didinium, in fact, so quickly exterminated the paramecia in groups *A II* and *B IV* that it was necessary to omit the records of paramecia in the table of sequence of these infusions (cf. table 2). In *B II* also the paramecia cycle was considerably aborted by Didinium. Among other instances of a similar nature, the destruction of hosts of Colpoda by the suctorian Podophrya may be mentioned. In other words, one who closely follows a series of infusions day by day cannot but be impressed with the intense struggle for food and the eternal warfare in this microcosm, and become con-

<sup>17</sup> Biological Bulletin, vol. 22, no. 1, 1911.

vinced, though he cannot prove, that in the final analysis the paramount factor is food, though many other factors, such as excretion products, etc., may play a not unimportant part. Biometrical study of variation in certain Protozoa shows that the average size of the population is smaller after their period of greatest abundance in an infusion and that "there can be little doubt that one of the chief factors which induce saprophytes like *Chilomonas* to disappear from a culture is that the medium no longer furnishes proper food (either in amount or kind, or both)."<sup>18</sup>

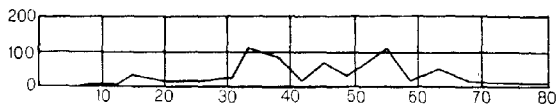


Fig. 14 B1 group. Hypotrichous fauna at the bottom.

#### VIII. CONJUGATION

Comparatively few epidemics of conjugation were observed in this entire study, and these were chiefly among paramaecia, so that the data in this connection are quite meager. It therefore has not been possible to make any definite correlations between the presence of the phenomenon and the fate of the conjugating forms in the infusions. However, a study of the records in regard to *Paramaecium* seems to show that conjugation usually occurs when a comparatively large number of individuals are present and that immediately following an epidemic there is a temporary decline in the number of specimens observed. After this decline there may or may not be a large increase in the number of animals.

It seems clear that in many cases conjugation is coincident with sudden changes in the environment. In fact the phenomenon may occur in certain cases almost solely among individuals which have been carried to the bottom with falling zoogloea. But that this does not of necessity bring about conjugation is shown by infusions B1 and B2. Conjugating paramaecia were not seen at

<sup>18</sup> Pearl: Variation in *Chilomonas* under favorable and unfavorable conditions, *Biometrika*, vol. 5, 1906-1907.

the surface of either of these infusions, and at the bottom it was only observed in *B1*. At the point marked x (fig. 12) fully 95 per cent of the animals were conjugating. Nevertheless the paramecia fauna at the bottom ran practically the same course in each infusion, in fact it survived somewhat longer in the infusion in which conjugation was not observed. This culture also illustrates a case in which a temporary decline in numbers occurred immediately after an epidemic of conjugation (cf. fig. 12).

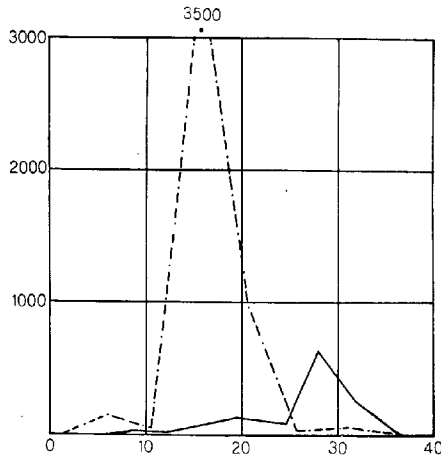


Fig. 15 *B IV* group. Vorticella fauna at the surface (---) and bottom (—).

In group *B III*, in which there was an exceptionally large bottom fauna, conjugation was observed among the paramecia at the surface and bottom simultaneously, but was somewhat more prevalent at the top. Fig. 13 shows that the epidemic occurred at the period of the surface maximum and that the ensuing decline at the top was coincident with a remarkably large increase in the bottom growth.

Apparently many species of infusoria do not resort to conjugation to sustain rapid cell division when the environment is slowly changing and the data give no reason for believing that

conjugation effects 'rejuvenation.' In many cases encystment occurs and the organisms remain at the bottom when conditions become somewhat unfavorable; but undoubtedly the majority die after their period of maximum abundance. My experience with these cultures leaves me with the impression that conjugation will be found to be a means resorted to by many species to survive acute changes in the environment, which, for example, preclude encystment. It is suggestive in this connection that in forms like the hypotrichida, which, as is well known, have a decided tendency to encyst, and the cysts of which were observed in great abundance at the bottom of these infusions after the active forms passed their maximum, not a single syzygy was observed; while in *Paramecium*, in which the power of encystment has never been established, conjugation is recorded comparatively frequently. However, it is also clear from this work that a condition which will induce conjugation in one race of *Paramecium* will not always induce it in another, as epidemics have occurred between small races, while among giant races intermingled with them syzygies were not seen. This, of course, is in accord with Jennings' studies on *Paramecium*.<sup>19</sup> The problem of the conditions inducing conjugation, and also of the effect of conjugation has recently become so complex from our increasing knowledge of the life history of various *paramecium* genotypes, that the observations here recorded are interesting chiefly as throwing a side light on certain factors of the phenomenon as they appear in large cultures.

#### IX. SUMMARY

The following points<sup>§</sup> may be emphasized:

1. Ordinary hay added to tap water will not produce an infusion which is productive of a sufficient number of representative protozoan forms to make it profitable for the study of protozoan sequence.
2. Air, water, and hay are all sources from which the protozoa of infusions are derived, and increase in importance in the order

<sup>19</sup> What conditions induce conjugation in *Paramecium*? Jour. Exp. Zool., vol. 9, no. 2, 1910.

given. Of these three, however, air is practically a negligible factor in seeding infusions.

3. In hay infusions, seeded with representative forms of the chief groups of Protozoa, there is a definite sequence of appearance of the dominant types at the surface of the infusion, i.e., Monad, Colpoda, Hypotrichida, Paramecium, Vorticella and Amoeba.

4. The sequence of maximum numbers and of disappearance is identical with that of appearance, except that apparently the position of Amoeba advances successively from the last (sixth) place to the fifth place and then to the fourth place.

5. A definite sequence of forms is not apparent at the middle or bottom of the infusions.

6. The middle of the infusions is tenanted chiefly by a free-swimming population brought there by an overcrowding at the top or bottom.

7. All of the protozoan forms considered (except Amoeba) are chiefly surface dwellers and it is evident that when they pass their greatest development at the surface this maximum is seldom approached again, and their cycle is practically over.

8. The major rise and fall in numbers are usually about equally rapid, though the final disappearance of an organism may be long deferred.

9. The appearance of any of the protozoan forms under consideration (excepting Amoeba) in appreciable numbers at the bottom is most often coincident with or immediately subsequent to its surface maximum, and portends its more or less rapid elimination as an important factor in the life of the infusion.

10. Numerous abnormal individuals and cysts are frequently to be found at the bottom in great abundance immediately after the surface maximum.

11. There is some evidence that amoebae migrate from the surface to the bottom which is their chief abode.

12. The observations give the impression that some amoebae appear as amoeba-flagellates which gradually increase in size and finally assume the form of typical *A. proteus*.



13. There is some evidence that the relative potential of division of the various forms may have an appreciable influence on the sequence of the maxima.

14. Emphasis is put upon the strictly biological interrelations (e.g., those involving food and specific excretion products) of the various forms as the most important determining factors in the observed sequence.

15. The observations suggest that conjugation will be found to be a means resorted to by many species to survive acute changes in the environment, which, for example, preclude encystment.

# CHEMICAL PROPERTIES OF HAY INFUSIONS WITH SPECIAL REFERENCE TO THE TITRATABLE ACIDITY AND ITS RELATION TO THE PROTO- ZOAN SEQUENCE

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## FIVE FIGURES

In the preceding paper<sup>1</sup> are presented the results of a study of the succession of the protozoan fauna of a series of hay infusions. The present paper gives the results of some chemical investigations on the infusions employed by Professor Woodruff with a view to correlating, if possible, certain chemical conditions with the protozoan sequence there shown to occur.

Although one cannot hope to obtain a complete analysis of the chemical factors involved, there are a limited number of determinations which can be made with some degree of ease and accuracy, and which may quite reasonably be expected to throw light upon this problem. In the present work a preliminary survey was made to determine those estimations which would be likely to yield satisfactory results, with the view of instituting an intensive study of such factors. The determinations<sup>2</sup> thus first made were: (1) Phenolphthalein acidity, (2) Methyl-orange alkalinity, (3) Oxygen consumed, (4) Chlorides, and (5) Solids (total, organic and inorganic).

The 'phenolphthalein acidity' was obtained by titrating 5 cc. of infusion with 0.01 N NaOH, using phenolphthalein as indicator.

<sup>1</sup>L. L. Woodruff: Jour. Exp. Zool., vol. 12, No. 2. The present work was undertaken at the suggestion of Professor Woodruff, to whom I am indebted for suggestions and criticism.

<sup>2</sup>Cf. A. W. Peters: Amer. Journ. Physiol., vol. 17, p. 454, 1907.

For the 'methyl-orange alkalinity' 5 cc. of infusion were titrated with 0.01 N HCl with methyl-orange as the indicator. As suggested by Peters,<sup>3</sup> the samples were titrated under xylol, thus retarding the loss of volatile matter during the process. In this manner was obtained the *titratable acidity* which, however, as Peters<sup>1</sup> points out, is probably not a correct expression of the concentration of  $H^+$  ions with which the organisms are in contact. For the third and fourth determinations recourse was had to methods employed in sanitary water analysis. To determine the oxygen consumed, 5 cc. of infusion, filtered clear, and diluted to 200 cc. with distilled water, were treated with 10 cc. of 50 per cent  $H_2SO_4$  and 0.01 N  $KMnO_4$ , the excess of the permanganate being titrated back with 0.01 N oxalic acid. For the chloride determination, 5 cc. infusion, filtered clear, were titrated with 0.01 N  $AgNO_3$ , using a solution of  $K_2CrO_4$  as an indicator. Total solids were obtained by evaporating 25 cc. of the filtered infusion to dryness. The residue was ignited, thus furnishing the data for the inorganic solids. The difference between these two last values is the solid organic matter.

The three latter determinations are recorded in table 1. Infusion 2 suggests a general increase in oxidized material. The data, however, are scant and lack uniformity. The figures for the chlorides show a rise to a maximum with a subsequent fall. What significance, if any, may be placed upon these data it is impossible to say. The ratios  $\frac{\text{inorganic matter}}{\text{organic matter}}$  indicate, as is to be expected, a general trend toward mineralized material. Of all the preliminary data, those for 'phenolphthalein acidity' appeared to be most characteristic and constant. Moreover this determination lent itself very readily to serial estimation. It was therefore planned to study this factor in considerable detail for a large number of infusions prepared in various ways.

<sup>3</sup> Loc. cit., p. 463.

<sup>4</sup> Loc. cit., p. 464.

TABLE 1

PRELIMINARY INFUSION 1*										PRELIMINARY INFUSION 2*									
AGE DAYS		Cubic centimeters 0.01 N $\text{AgNO}_3$ per 100 cc. Infu- sion	CHLORIDES per 100 cc. Infu- sion	SOLIDS						Cubic centimeters 0.01 N $\text{AgNO}_3$ per 100 cc. Infu- sion	CHLORIDES per 100 cc. Infu- sion	Cubic centimeters 0.01 N $\text{KMnO}_4$ per 100 cc. Infu- sion	OXYGEN CONSUMED per 100 cc. Infu- sion	SOLIDS					
				Grams per 100 cc. Infusion										Grams per 100 cc. Infusion					
				Inorganic			Organic							Inorganic			Organic		
				Total	Inorganic	Organic	Total	Inorganic	Organic					Total	Inorganic	Organic	Total	Inorganic	Organic
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presence, initially, of as large a number of representative protozoan forms as possible.

*Atypical member.* Infusion D-1 was prepared exactly as the above with the exception that the water to which the boiled hay and infusion were transferred had been heated to about 90° C. and no protozoa were introduced. By this means was obtained a protozoön-free culture fluid.<sup>7</sup>

*Series B: Typical members.* Infusions B-1, B-2, B-21, B-22, B-31, B-32, B-41, and B-42<sup>8</sup> were prepared by boiling 20 grams Timothy hay for five minutes, just as in series A, and then straining into unboiled water so that a final volume of 5 liters was obtained. These were seeded exactly as in series A.

*Atypical members.* Infusion E-1 was prepared just like the typical members of this series except that the water into which the boiled infusion was strained had been raised to a temperature of 90° C. We have thus a protozoön-free infusion of series B, just as D-1 is a protozoön-free infusion of series A. Infusions BB-1, BB-2, BB-3, BB-4, BB-5, and BB-6 were prepared exactly like the typical members of this series but in addition were treated in various ways. BB-1 and BB-2 were left unchanged, serving as controls. To BB-3 and BB-4 were added 5 and 20 grams of dextrose respectively. BB-5 was kept practically neutral to phenolphthalein by adding, when necessary, the calculated amount of NaOH. This necessitated stirring at each addition of alkali, and hence, as a check, BB-6 was stirred at the same time.

*Series C: Typical members.* Infusions C-1, C-2, C-3, C-31, C-41, C-42, and M-1 each consisted of 20 grams unboiled hay with 5 liters unboiled water. To this was added a small amount of seed. With certain exceptions, mentioned elsewhere, the hay was kept continuously at the bottom of the infusion.

*Atypical member.* Infusion S-1 was prepared by heating 20 grams of dry hay in an autoclave and adding 5 liters of water which had been warmed to 90° C. S-1 is therefore a protozoön-free infusion of series C.

<sup>7</sup> No attempt was made subsequently to keep the infusion free from bacteria.

<sup>8</sup> Infusions B-41 and B-42 were subjected to a chemical examination so infrequently that the results are omitted from table 3.

*Bacterial infusions.* Eight infusions were prepared as follows: In each of eight cotton plugged flasks, 2.8 grams of hay and 700 cc. of water were placed and the mixtures sterilized in an autoclave. They were allowed to cool and were then treated in various ways: two were kept sterile; four were inoculated with a pure culture of *B. coli*; and two were inoculated with a pure culture of *B. subtilis*.

On the foregoing cultures records were obtained for the 'phenolphthalein acidity' and 'methyl-orange alkalinity.' In the ideal experiment, the temperature should have been maintained constant throughout the period during which the infusions were under observation; or, at least, all infusions should have been subjected to the same changes in temperature. Neither of these conditions could be conveniently brought about.<sup>3</sup>

#### RESULTS

The data secured in this study may be most readily presented by tables and curves. In tables 2, 3, 4, and 5 are recorded the results of the 'phenolphthalein acidity' and 'methyl-orange alkalinity' determinations, expressed in cubic centimeters of 0.01 N NaOH or 0.01 N HCl per 100 cc. of infusion. Peters has made the observation that the acidity becomes greater as the depth of the infusion increases and in order to give this quantitative expression, titrations were made on samples taken from the bottom of the infusion at frequent intervals during its history. These results are given in italics immediately above the figures for the top, and are expressed as so many cubic centimeters of 0.01 N NaOH or 0.01 N HCl per 100 cc. of infusion *greater* (or *less*) than the titrations for samples taken from the top. The principal points of interest, brought out in these tables, are illustrated in figs. 1, 2, 3, and 4. In calculating average curves only the 'typical members' of the three series were included. As a matter of fact, as far as the actual titrations are concerned, some of the 'atypical members' might have been included, e.g., D-1, E-1 and S-1

<sup>3</sup> For a discussion of the influence of temperature on these infusions, cf. Woodruff, loc. cit., p. 248 and also p. 274 of the present paper.









TABLE 5  
*Methyl Orange Alkalinity*  
(Cubic centimeters of 0.01 N HCl per 100 cc. infusion)

[illegible]

do not differ materially from the 'typical members' of their respective groups. From the average of series *C* in addition to the 'atypical member' S-1, certain 'typical members' C-1, C-3 and M-1 were also omitted—C-1 because on the fifth day it was slightly stirred; C-3 because during the early part of its history the hay was at the top; and M-1 because it was stirred at certain intervals for a definite purpose, as explained in another place.

In boiled infusions (series *A* and *B*) we can corroborate Peters'<sup>10</sup> results in regard to the 'phenolphthalein acidity,' i.e., a rapid rise in which a maximum is reached in from two to six days, followed by a more gradual decline; the lowest point being reached fifteen to twenty days earlier in series *B* than in series *A* (fig. 1). The curve for series *C* is somewhat different; a maximum being attained much more gradually. Further reference will be made to this below.

<sup>10</sup> Peters; Amer. Journ. Physiol., vol 18, p. 330, 1907.

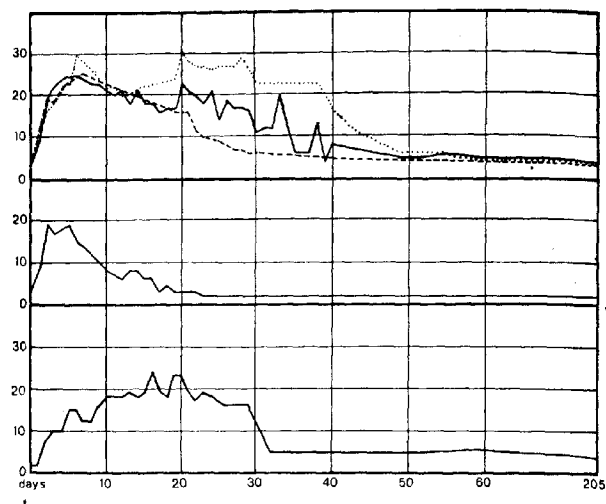


Fig. 1 'Phenolphthalein acidity' for infusions of series A, B, and C; samples taken from top of media. Ordinates represent number of cubic centimeters of 0.01 N NaOH per 100 cc. of infusion. *Upper curves:* series A, average acidity = ———; maximum acidity = .....; minimum acidity = - - - - -. *Middle curve:* series B, average acidity. *Lower curve:* series C, average acidity. Curves for maximum and minimum acidity of series B and C are not given as the variations are not important.

It would seem that for the 'phenolphthalein acidity' at least, the inconstant temperature was not an important consideration. Infusions of the entire series A (infusions started at different times and therefore experiencing different temperature changes) show no greater individual variations in acidity than does the group A-1 . . . . . A-6 (infusions started on the same day and hence subjected to the same changes in temperature). This is illustrated in table 2. For this reason, when calculating average curves, I have not considered it necessary to exclude infusions merely because they were subjected to different temperature variations.

As to the factors underlying the production of acid, I agree with the view of Peters<sup>10</sup> viz., that bacterial fermentation mainly is

responsible. That the protozoa play a relatively small part in the acid production is shown in infusions D-1, E-1, and S-1, in which throughout their history practically no protozoa were present, and yet their acidity curves do not differ materially from the others of their respective series (tables 2, 3, 4).

In order further to demonstrate that the bacteria are almost entirely responsible for the acid production, hay infusions were prepared and inoculated with pure cultures of bacteria as described on page 269. The results for 'phenolphthalein acidity' are recorded in table 6 (also fig. 5). It is apparent (especially for the *B. coli* infusions) that the acidity curves do not show any striking variations from those obtained from cultures promiscuously seeded with various forms of protozoa and bacteria.

TABLE 6  
*Phenolphthalein acidity. Bacterial infusions*  
(Cubic centimeters 0.01 N NaOH per 100 cc. infusion)

	DAYS															
	0	1	2	3	4	5	6	7	8	9	10	11	12	19	28	48
Sterile-1.....	4		4						4				7	6	6	7
Sterile-2.....																
Coli-1.....	4	18	18	16	13			11	10				8	8	8	5
Coli-2.....		21	16	14	13			10	9				7	7	6	5
Coli-3.....		19	23	24	22			22	20				20	19	17	10
Coli-4.....			20	21	20			20	19				17	16	14	7
Subtilis-1.....	4	12	10	9	7	6		5					6	6	6	6
Subtilis-2.....		8	10	8	7			6	5				6	6	7	6

From figs. 2 and 3, it is evident that the maximum difference in top and bottom acidity for series *A* is considerably less than the minimum difference for series *C*. Fig. 4 illustrates the maximum difference in series *C*. From table 3 it is apparent that in series *B* there is no essential difference between top and bottom acidity.

The difference between acidity at the top and bottom of the infusion is probably to be referred to the unequal bacterial food supply in these two regions. In cultures of series *B* the food supply is uniform throughout the media, and hence acidity differences are not apparent. Where, as in infusions of series *A*,

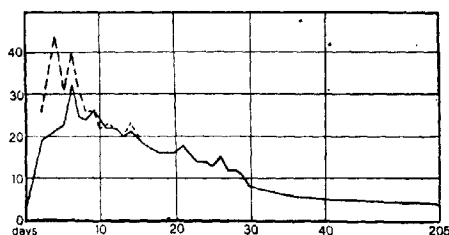


Fig. 2 Infusion A-2, illustrating *maximum* difference in cultures of *series A* between 'phenolphthalein acidity' of top and bottom of infusion. Ordinates represent number of cubic centimeters of 0.01 N NaOH per 100 cc. of infusion. Top = —————; bottom = - - - - -.

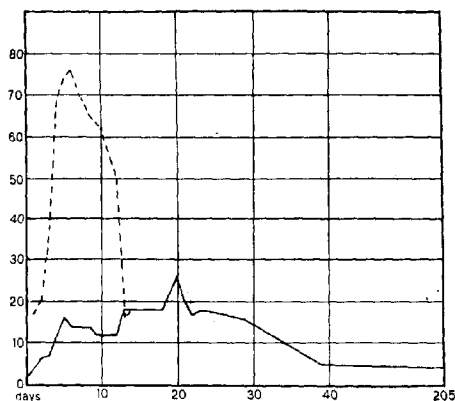


Fig. 3 Infusion C-2, illustrating *minimum* difference in cultures of *series C* between 'phenolphthalein acidity' of top and bottom of infusion. Ordinates represent number of cubic centimeters of 0.01 N NaOH per 100 cc. of infusion. Top = —————; bottom = - - - - -.

at the bottom is hay, whose constituents are dissolving continuously, differences of this nature are manifest. However, in the course of preparation, a considerable portion of material has already entered solution, equilibrium is thus quickly reached and acidity differences—never very great—soon disappear. The infu-

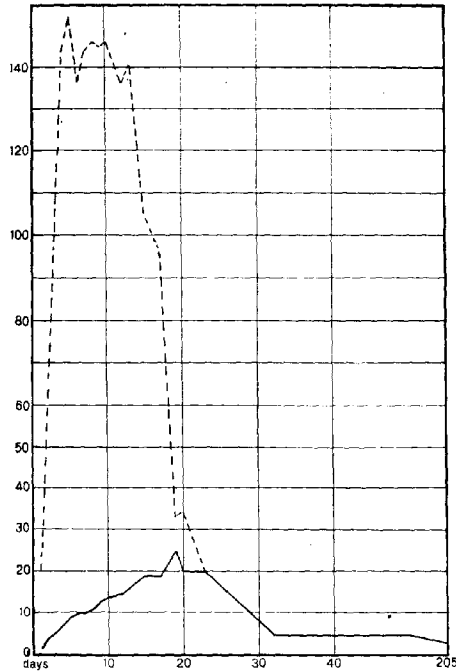


Fig. 4 Infusion C-31, illustrating *maximum* difference in cultures of series C between 'phenolphthalein acidity' of top and bottom of infusion. Ordinates represent number of cubic centimeters of 0.01 N NaOH per 100 cc. of infusion. Top = ———; bottom = - - - - -.

sions of the *C* series present quite a different condition. Here untreated hay is covered with clear water. There is the opportunity for the formation of a concentrated solution of hay constituents at the bottom, which serve for the development of a high degree of acidity. During the first few days there is a relatively concentrated solution at the bottom with a corresponding high acidity, while at the top of the infusion both these conditions are reversed. As diffusion proceeds, the concentration approaches uniformity and hence acidity differences tend to disappear. Uni-

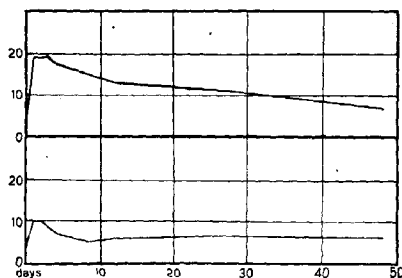


Fig. 5 Bacterial infusions, average 'phenolphthalein acidity.' Ordinates represent number of cubic centimeters of 0.01 N NaOH per 100 cc. of infusion. *Upper curve, coli. Lower curve, subtilis.*

form acidity is of course attained when the rate of diffusion of hay constituents balances that of their solution. The present study contains many data illustrative of the above explanation.

Peters is of the opinion that the deeper layers of the infusion owe their higher acidity to greater concentrations of  $\text{CO}_2$ . "Owing to the slowness of diffusion, each upper layer of the liquid protects the adjacent lower layer from rapid interchange with the air and enables the deeper layer to maintain a higher acidity."<sup>11</sup> This factor is, I believe, probably only of secondary importance. The greater acidity in the lower regions of the hay infusion is to be attributed primarily to their greater concentration of acid-yielding hay constituents, as explained above. Peters' explanation would not entirely account for the fact that infusions of the *B* series show uniform acidity throughout the cultures, while the acidity differences in an infusion of series *C* is very great.

In the early history, at least of the typical members of series *C*, the increase in acidity is not uniform with increase in depth. Until the immediate region of the hay is reached, the acidity may be practically uniform. To illustrate this: on the fifth day a sample taken from just above the hay in infusion C-2<sup>12</sup> titrated exactly the same as a sample from the top, while the difference between the acidity at the top and extreme bottom was 58. On

<sup>11</sup> Loc. cit., p. 332.

<sup>12</sup> See table 4.

the sixth day a sample taken from just above the hay in infusion (C-31)<sup>12</sup> titrated 20, against 136 an inch below (in the midst of the hay), and 10 at the top of the infusion. It is thus clear why the tops of typical infusions of series C are so tardy in reaching their maximum acidity (fig. 1).

Infusion M-51<sup>12</sup> on the second day of its history was showing an acidity difference of 41 between the top and bottom. It was then thoroughly stirred after which samples from both top and bottom titrated 20. By the next day an acidity difference had again developed to the extent of 20. It was again thoroughly mixed (both top and bottom then titrating 29), and two days later, an acidity difference (to the extent of 9) was once more apparent. From here on, however, equilibrium was soon reached. This further illustrates the point made that acidity does not increase uniformly with the depth. On the second day of this infusion the top and bottom titrated 15 and 56 respectively. When thoroughly mixed the infusion gave a uniform titration of 20 instead of the average 35.

In the early history of the typical infusion of series C, the liquid in the immediate region of the hay is highly colored, while the liquid above is almost colorless. The approach to equality in acidity can be followed roughly by the gradual diffusion of this colored material.

The observations on acidity differences may be summarized as follows:

1. The greatest acidity is found near the source of supply of soluble material upon which the bacteria can act.
2. The difference between top and bottom acidity depends upon the relative concentrations of acid-yielding hay constituents in these regions.
3. When there is uniform concentration, or when diffusion keeps pace with solution, there is no essential difference between top and bottom acidity.

From table 5 we see that the curve for 'methyl-orange alkalinity' follows an irregular course with a general upward tendency, as also noted by Peters. The latter's figures for these determinations are from three to ten times as great as here recorded. This



may possibly be accounted for by the difference in the quality of the tap waters used in the two investigations. For example, the tap water employed in our experiments titrated 6 cc. 0.01 N HCl per 100 cc. of sample, while water from a neighboring well titrated 22 cc. per 100 cc. of sample.

#### RELATION OF TITRATABLE ACIDITY TO PROTOZOAN SEQUENCE<sup>13</sup>

The organisms make their appearance at about the same time in the three types of infusions. The protozoa of the *B* series run their course more rapidly than those of the *A* and *C* series, the two latter not varying markedly in this respect. These time relations are probably in great part dependent upon the food supply. The latter is the determining factor for acidity. However, the degree of titratable acidity existing in an ordinary hay infusion has no important bearing on the duration and character of the cycle taking place, for in the three types of infusions, the sequence of the dominant protozoan forms studied was the same, although these culture fluids show characteristic differences in the degree of acidity. Furthermore, the cycle normally occurring in the *B* series was not influenced when the acidity was purposely increased to two or three times that ordinarily obtained.<sup>14</sup> When, however, the acidity was raised to seven or eight times that of the typical *B* infusion,<sup>14</sup> the development of the protozoan fauna was greatly retarded. Decreased acidity, likewise, has no appreciable influence upon the protozoan cycle: BB-5 had an almost negligible acidity<sup>15</sup> throughout its history, yet the sequence of organisms did not differ in any important respect from that found in the control infusion, BB-6, or other typical infusions of this series.<sup>16</sup>

While certain species of protozoa are particularly abundant during the period of high acidity and others during low acidity,

<sup>13</sup> Cf. Woodruff, loc. cit.

<sup>14</sup> This was accomplished by adding dextrose to the infusion. Cf. p. 268 and table 3.

<sup>15</sup> Brought about by adding alkali when necessary. Cf. p. 268 and table 3.

<sup>16</sup> The biological examination of infusions BB-1 . . . BB-6 was made by Dr. Woodruff but not reported in his paper.

detailed correlations are not possible. It is my opinion from this study that even did we know exactly the course of the curves illustrating mineralization, oxygen consumed, acidity, alkalinity and other information of a general character—with such data alone nothing but the most superficial correlations could be expected. It is not improbable that a partial explanation of the sequence of protozoa occurring in hay infusions may be found in the influence of certain excretory products of an organism upon others of its own or other species. That this is a consideration of some importance is brought out in a recent paper by Woodruff,<sup>17</sup> in which it is shown that the excretory products of paramaecia inhibit the reproduction of these organisms.

#### SUMMARY

The acidity of hay infusions is essentially due to bacteria, their efficiency in producing acid being governed by the concentration of the infusion in acid-yielding materials. The protozoa play a relatively small part in the production of acid. The sequence of protozoa and the course of the titratable acidity possess no intimately mutual relation. Either may vary within wide limits without appreciably influencing the course of the other.

<sup>17</sup> Woodruff: Jour. Exp. Zool., vol. 10, p. 557. 1911.



## STUDIES IN THE LIFE CYCLE OF HYDATINA SENTA

### III. INTERNAL FACTORS INFLUENCING THE PROPORTION OF MALE-PRODUCERS<sup>1</sup>

A. FRANKLIN SHULL

#### SIX FIGURES

Introduction.....	283
Observations and experiments.....	284
Decrease in the proportion of male-producers with long-continued par- thenogenesis.....	284
Decrease in size of family with long-continued parthenogenesis.....	288
Effect of inbreeding on the proportion of male-producers.....	291
Effect of long duration of the fertilized egg stage on the proportion of male-producers.....	296
Period at which the nature of a female is determined.....	301
Discussion.....	308
Summary.....	315
Bibliography.....	316

#### INTRODUCTION

The existence of internal<sup>2</sup> factors affecting the life cycle of *Hydatina senta* was demonstrated in my earlier experiments (Shull, '11 a) in which two or more parthenogenetic lines of rotifers were bred under the same external conditions, and yielded different proportions of male-producers. Two such lines were crossed, and several new lines thus produced. One of these new lines was crossed back to one of the parent lines, and another new line

<sup>1</sup> Contributions from the Zoological Laboratory of the University of Michigan, No. 138.

<sup>2</sup> In a recent suggestive paper, Woltereck ('11) has included me among those who hold that the life cycle of *Hydatina* is determined by external agents, notwithstanding that a large section of the paper which Woltereck cites (Shull, '11 a) was devoted exclusively to internal factors. In the hope of correcting this impression of Woltereck's which may be shared by others, the present article will be made to include only evidence regarding internal agents, leaving to a future paper the further work that has been done or is in progress on external agents.

derived from their union. These various lines reacted to the same environment by yielding different proportions of male-producers. Such differences could only be explained by assuming that each line possessed an internal nature different from that of the others.

The nature of the internal agent or agents was not discussed in connection with the earlier experiments, since what I hoped might prove a crucial test of their nature, namely, the results of inbreeding, was not available because the fertilized eggs obtained by inbreeding did not hatch. I have now obtained offspring by inbreeding, and the evidence from this source, together with that from the former crosses, serves at least to eliminate some possible conceptions of the internal agents. The inbreeding experiments are described and their bearing discussed in the following pages. The question whether the internal factor, whatever its nature, is constant, or whether it may undergo progressive change, is also taken up. And finally, experiments which seem to determine the time at which external agents may affect the cycle are described.

#### OBSERVATIONS AND EXPERIMENTS

##### *Decrease in the proportion of male-producers with long-continued parthenogenesis*

From observations incidentally made on several lines of rotifers bred through a considerable number of generations, I gained the impression that there was a progressive decrease in the proportion of male-producers with long-continued parthenogenesis. Such a condition, if proven to exist, besides being of considerable theoretical interest, would be of importance in judging the results of breeding experiments in which two distinct or unrelated lines are compared with one another. Inasmuch as a number of my experiments involved such a comparison, I have compiled data from a number of lines which were reared through a number of generations large enough to give results of value. There are eight of these lines, each including 46 or more generations, which are given in table 1.

TABLE 1

*Showing proportion of male-producers in eight parthenogenetic lines of Hydatina senta in successive generations. The generations are taken two by two, and the proportions are given in per cents.*

	GENERATION											
	1-2	3-4	5-6	7-8	9-10	11-12	13-14	15-16	17-18	19-20	21-22	23-24
Line 1.....	42.2	49.1	66.2	75.9	64.0	31.8	5.1	47.4	77.3	64.2	36.4	18.9
Line 2.....	19.1	12.5	28.1	0.0	26.4	62.5	25.4	79.2	20.4	4.4	0.0	32.2
Line 3.....	60.0	69.5	50.0	48.7	6.8	42.1	12.2	30.3	33.8	0.0	42.3	36.3
Line 4.....	11.2	27.4	1.7	1.4	27.8	9.5	7.1	7.0	5.0	8.7	45.5	10.3
Line 5.....	45.2	29.8	47.0	25.4	54.2	53.2	49.4	33.3	37.2	34.8	9.0	48.7
Line 6.....	0.0	4.0	0.0	1.1	10.2	35.4	0.0	0.0	0.0	17.6	4.9	2.9
Line 7.....	1.4	18.9	0.0	2.2	1.2	2.0	45.8	2.6	27.1	1.3	9.6	1.9
Line 8.....	31.3	56.1	33.6	15.8	9.2	11.6	0.0	7.8	14.2	18.6	1.6	8.7
Average.....	26.3	33.4	28.3	21.3	25.0	31.0	18.1	26.0	26.9	18.7	18.7	20.0

	GENERATION											
	25-26	27-28	29-30	31-32	33-34	35-36	37-38	39-40	41-42	43-44	45-46	
Line 1.....	4.5	0.0	2.5	8.4	6.8	3.4	0.0	9.5	48.5	26.3	66.2	
Line 2.....	17.5	52.3	17.5	14.9	25.3	12.0	4.5	15.3	5.5	0.0	4.6	
Line 3.....	34.0	42.8	24.4	23.8	45.5	40.6	32.3	38.9	34.4	50.0	3.4	
Line 4.....	27.9	53.6	43.0	62.6	13.0	16.3	17.6	15.1	3.3	17.0	2.6	
Line 5.....	29.0	19.3	0.0	13.3*	40.8	18.4	22.9	43.1	16.2	10.9	25.0	
Line 6.....	11.8	22.4	13.6	5.4	14.2	17.8	0.0	5.0	23.4	8.0	3.4	
Line 7.....	52.0	10.2	0.0	9.6	6.6	31.5	0.0	0.0	1.5	0.0	0.0	
Line 8.....	16.9	10.6	25.0	4.2	34.1	1.7	2.8	7.8	12.1	0.0	0.0	
Average.....	24.2	26.4	15.8	17.8	23.3	17.7	10.0	16.8	18.1	14.0	13.2	

In compiling the data, I have combined the generations two by two, partly to save labor in computing percentages, partly to smooth out the great fluctuations that often occur from one generation to the next. Two methods of handling the data would have been practicable. All the male-producers and the female-producers, respectively, of corresponding generations in all eight lines could be added together, and the percentage of male-producers computed for the whole group. Or the percentages could be determined for each line separately, and then an average of the eight percentages in corresponding generations computed. The former method would give to a line producing large families much greater weight in determining the end result than to a line that produced small families; the latter method makes all the lines of equal importance, regardless of the number of individuals.

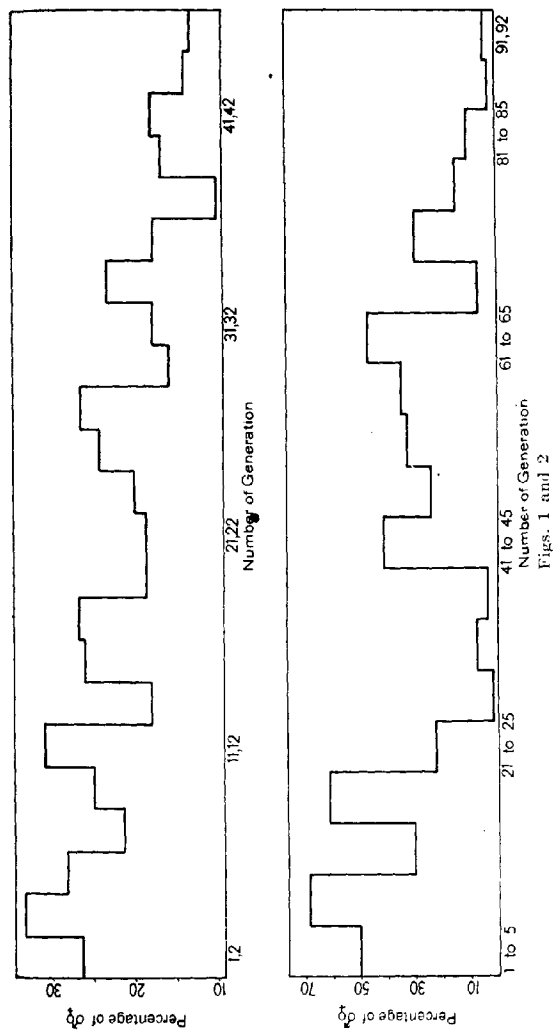
Since my incidental observations had been made on lines producing large families, it seemed more conservative to adopt the latter method, of computing the percentages in each line separately, and taking the mean of the eight percentages. This method has the further advantage, as we shall see later, of enabling us to analyze the result.

Most of the lines in table 1 included more than 46 generations. In such cases, the first 46 generations are given. In two cases the lines included 92 or more generations; these were divided into two parts, the first to the 46th generation constituting one line as given in table 1, the 47th to the 92d another line, while the remainder were omitted. Thus, lines 1 and 2 are parts of the same line, as are also lines 6 and 7. Generations not fully recorded have been omitted, except where they were necessary (as they were in one case) to complete the 46 generations. The one case of incomplete records can hardly invalidate the results.

These lines have been, for the most part, recorded in detail in my former papers (Shull, '10, '11 a), or are given in the following pages. Lines 1 and 2 are found almost complete in table 1 of those papers, line 3 in table 3 (right column), line 4 in table 34 (right column), line 5 in part in table 37 (middle column), lines 6 and 7 almost complete in tables 20 and 34 (left column in both), and line 8 in part in tables 2 and 6 of this paper. As the details of the families may be had in the places cited, they are not repeated here.

The mean percentages of male-producers in the various pairs of generations, given at the bottom of table 1, show great fluctuations; but the earlier generations have plainly more male-producers than the later generations. When they are represented by a curve, as in fig. 1, it is obvious at a glance, notwithstanding the fluctuations, that there is a progressive decrease in the proportion of male-producers from the first generation to the last.

If the eight lines be examined separately, it is seen that most of this decrease in the proportion of male-producers is due to three lines (1, 2, and 5), and perhaps a fourth (line 8). Lines 1 and 2, which are two parts of the same line, are plotted in fig. 2. Because we have here only a single line, the fluctuations are so



Figs. 1 and 2



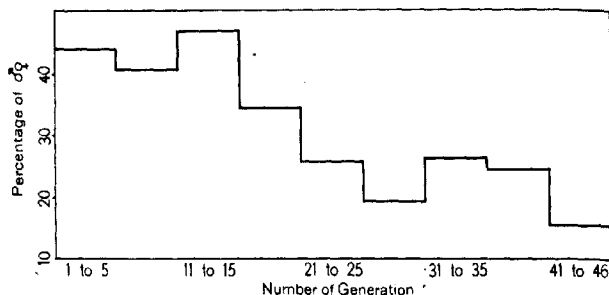


Fig. 3

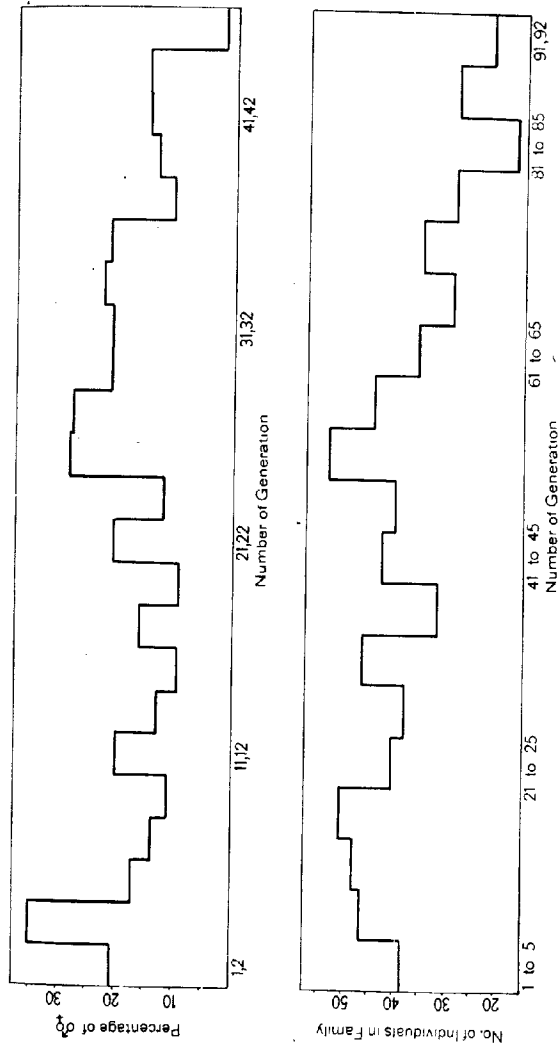
great that I have taken the generations in groups of five. Despite the enormous differences among the groups of five generations, there is seen to be a general diminution in the proportion of male-producers through the series. In like manner, line 5 $\frac{1}{2}$  (fig. 3) shows such a decrease in a most unmistakable manner.

The remaining five lines, including line 8 which would show a considerable decrease in the early generations, are combined and plotted in fig. 4. Here it would perhaps not be justifiable to assume a decrease in the proportion of male-producers in the lines as a whole. A possible explanation of these differences between different lines, and the importance of the phenomenon of decrease in the proportion of male-producers where it occurs, are discussed elsewhere.

*Decrease in size of family with long-continued parthenogenesis*

It became apparent in several lines of rotifers that the families became gradually smaller, and that it was increasingly difficult to maintain the animals in a healthy and vigorous condition. To determine whether there were any possible connection between the diminution in the size of family (reduction of vigor) and the decrease in the number of male-producers, I have plotted the size of family in those lines that showed the decrease in the proportion of male-producers.

For the sake of comparison, the generations are again taken in groups of five. Fig. 5 represents lines 1 and 2 of table 1, and may



Figs. 4 and 5

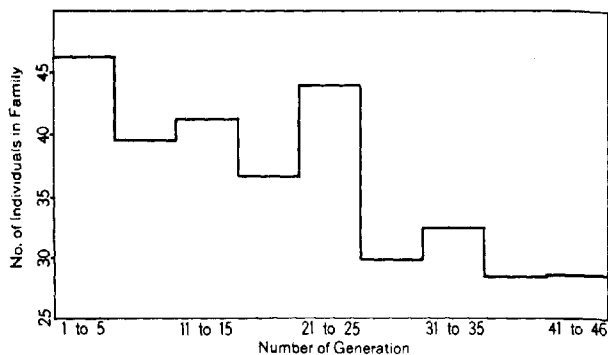


Fig. 6

be compared with fig. 2, which shows the proportion of male-producers for the same lines. Fig. 6 represents line 5 of table 1, and is to be compared with fig. 3. Both curves show a decrease in size of family, which is especially marked in fig. 6. It does not follow, however, that there is any relation between the two phenomena, namely, the decrease in the size of family and the decrease in the proportion of male-producers with long-continued parthenogenesis.

To discover whether the great fluctuations in the proportion of male-producers has any relation to size of family, several lines that showed the greatest fluctuations have been examined in detail. While in some cases the families, or groups of families, showing a great increase in the proportion of male-producers over the preceding generations also showed a great increase in the size of the family, this was not true in a number of other cases. One need not conclude from this that there is no relation between size of family (vigor of line) and proportion of male-producers. There are many accidents which might happen to a female whose family includes many male-producers. I have known a number of females to die as a result of accidentally (?) starting to devour a large *Paramecium* or a fiber in the water. The small families produced by such females may have included mostly male-producers, so that a study of individual families can hardly be

expected to show a correspondence between 'vigor' and the proportion of male-producers.

*Effect of inbreeding on the proportion of male-producers*

In the second of these studies (Shull, '11 a) I described experiments in which two distinct lines of rotifers, yielding different proportions of male-producers, were crossed, the zygotes giving rise to lines in some crosses yielding more male-producers than either parent line, in other crosses a proportion of male-producers intermediate between those of the parent lines. In order to explain these phenomena, an attempt was made to inbreed the same lines that were used in the earlier crossing experiments. Females were paired with males of the same line, and a large number of fertilized eggs was secured. These eggs, however, did not hatch before it was necessary to discontinue the experiments. I have now, however, in other lines, succeeded in obtaining viable offspring from females paired with their own nephews or cousins, and give the results in the following experiments:

*Experiment 1. Inbreeding.* Some winter eggs were collected in the spring and kept in an ice-chest or in cold running water until September. The eggs were then brought to room temperature and began to hatch in five days. From one of the females thus obtained was reared the line of rotifers used in this and the following inbreeding experiments.

Between October 7 and October 20, many females of the parthenogenetic line just mentioned were paired with males of the same line. Of 1099 eggs obtained, one hatched October 22, and from her another parthenogenetic line was bred. The number of male- and female-producers in this inbred line is compared, in table 2, with the corresponding data from that part of the original line which was reared at the same time.

It appears from the table that the inbred line yielded 16.7 per cent of male-producers, the original line only 10.6 per cent. In such an experiment, however, the original line is necessarily further removed from the fertilized egg than is the inbred line. We have learned above that there may be a progressive decrease

TABLE 2

Showing the number of male- and female-producers in two lines of *Hydatina senta*, one line being the result of inbreeding in the other line, that is, being derived from the offspring of a male and female both from the other line. Male-producers are designated ♂ ♀, female-producers ♀ ♀.

ORIGINAL LINE			INBRED LINE		
Date of first young	Number of ♂ ♀	Number of ♀ ♀	Date of first young	Number of ♂ ♀	Number of ♀ ♀
<i>October</i>			<i>October</i>		
24	1	8	24	0	28
26	4	30	25	10	36
28	0	28	27	2	21
29	0	48	29	2	17
31	5	40	30	2	43
<i>November</i>			<i>November</i>		
2	0	19	1	3	44
4	1	15	3	7	38
6	7	33	5	2	45
8	8	38	6	2	44
10	9	36	8	0	26
12	0	22	10	14	36
14	1	38	12	13	9
16	2	46	14	6	37
18	5	27	16	3	30
20	8	18	18	10	9
22	2	31	20	0	23
25	6	39	22	1	11
26	2	28	24	4	10
28	5	23	26	1	7
30	4	4	28	9	25
<i>December</i>			30	0	41
2	0	27	<i>December</i>		
4	3	40	2	2	33
7	3	23	4	1	43
9	11	4	6	20	20
12	1	22	9	11	10
13	0	5		17	2
	0	29*	11	2	13
15	0	15*	13	0	17
			15	3	14*
Total.....	88	736		147	732
Per cent of					
♂ ♀ .....	10.6		16.7		

\* Remainder of family not recorded.

in the proportion of male-producers, such that a comparison of two lines may show fewer male-producers in the older line, although the two lines would have been equal had each been taken at the same number of generations after the fertilized egg. An application of this discovery is found in the present experiment. The original line in this experiment is line 8 of table 1. It will be seen in that table that the early generations of the line had a larger number of male-producers than the subsequent generations. The original line passed through ten generations before the inbred line started. If these ten generations, comprising 147 male-producers and 315 female-producers, be added to the 27 generations given in table 2, then the total for the original line shows 18.2 per cent of male-producers, or a higher percentage than that of the inbred line. We may not be justified in including the first ten generations, as I have just done, but in view of the decrease in the proportion of male-producers in the later generations, it seems to me unsafe to ignore the early generations.

Incidentally it may be mentioned that the average size of family in the entire original line, exclusive of the last two families which were not fully recorded, was 34.5 as compared with 30.7 in the inbred line.

*Experiment 2. Twice inbreeding.* Females of the inbred line of the preceding experiment were paired with males of the same line. Of 144 eggs obtained, one hatched November 26, and became the parent of the line in table 3 designated 'twice inbred.' This line is compared with those parts of its parent (also inbred) line and the original ('grandparental') line that were reared at the same time.

Here the difference between the two inbred lines is not great, while the percentage of male-producers in the original line is considerably less. Since all three of the lines are at different 'ages,' that is, a different number of parthenogenetic generations has been passed through in each line since the fertilized egg from which the line was derived, it is of interest to note the proportion of male-producers in the whole lines, and not merely those parts that were bred simultaneously. In the original line, including 28 generations reared previously to the beginning of this experi-

TABLE 3

Showing number of male- and female-producers in three lines of *Hydatina senta*, the line in the second column being inbred from that in the first column, and the line in the third column from that in the second (see text)

ORIGINAL LINE			INBRED LINE		TWICE INBRED LINE			
Date of first young	Number of ♂♀	Number of ♀♀	Date of first young	Number of ♂♀	Number of ♀♀	Date of first young	Number of ♂♀	Number of ♀♀
November			November			November		
28	5	23	28	9	25	28	6	15
30	4	4	30	0	41	30	9	20
December			December			December		
2	0	27	2	2	33	2	7	39
4	3	40	4	1	43	4	2	18
7	3	23	6	20	20	6	2	31
9	11	4	9	11	10		8	26
12	1	22		17	2	9	1	23
13	0	5	11	2	13	11	5	8
	0	29*	13	0	17	13	1	16
15	0	15*	15	3	14*	15	0	3
							9	3*
Total.....	27	192		65	218		50	202
Per cent of ♂♀	12.3			22.9			19.8	

\* Remainder of family not recorded.

ment, there were 18.2 per cent of male-producers; and in the inbred line, including 19 earlier generations, 16.7 per cent. In the 'twice inbred' line, which is given in full in table 3, there were 19.8 per cent of male-producers.

It may be pointed out incidentally that in the original line as a whole, not including the last two generations which were incompletely recorded, the average size of family was 34.5, in the inbred line as a whole 30.7, and in the 'twice inbred' line 24.0. This seems to indicate a loss of vigor, perhaps due to inbreeding.

*Experiment 3. Inbreeding in another line.* In this experiment the comparative ages of the inbred line and the line from which it was derived are not known. The line designated A in table

TABLE 4

*Showing number of male- and female-producers in two lines of Hydatina senta, one line being derived from the other by inbreeding, as in table 2*

LINE A			INBRED LINE		
Date of first young	Number of ♂ ♀	Number of ♀ ♀	Date of first young	Number of ♂ ♀	Number of ♀ ♀
<i>June</i>			<i>October</i>		
17	7	14	12	35	16
19	11	17	14	23	25
21	20	22	17	0	21
22	25	11	18	33	16
23	25	24	20	1	11
25	19	9		30	22
26	21	14	22	43	8
27	29	19	27	0	16
			29	23	14
Total.....	157	130		188	149
Percent of ♂ ♀ .....	54.7			55.7	

4 was derived from a female taken from a culture in the laboratory about June 1. This culture originated from a collection of rotifers taken at Grantwood, New Jersey, in the latter part of March, 1911, and transplanted to manure cultures in Ann Arbor shortly afterwards. These manure cultures were maintained without observation until June, when the female that became the parent of line A was isolated.

Females of line A were paired with males of the same line about June 25 to June 27, and fertilized eggs obtained. These eggs were allowed to dry in the dishes and to remain dry until the last of August, when they were again covered with water. Females began to hatch from them in a week, and from one of them the 'inbred line' of table 4 was started. Completed records of this line were not kept until October 12, after which date eight complete generations were recorded.

The two lines in table 4 may not be of the same age; either one may be the older. Furthermore, since they were not bred simultaneously, the conditions may not have been the same. How-



ever, as the food cultures were frequently changed, each of these recorded lines must have been fed from three or more cultures. The averages of these three or more cultures should, I believe, be nearly equal. The evidence, though not complete, is presented for what it is worth. Line A included 54.7 per cent of male-producers, the inbred line 55.7 per cent.

Incidentally it may be pointed out that the average size of family in line A is 35.8, in the inbred line 37.4.

On the whole it seems that inbreeding does not markedly alter the proportion of male-producers, though perhaps had the inbred lines been bred as long as were the original lines, the decrease in the proportion of male-producers which sometimes accompanies long-continued parthenogenesis might have shown a slight decrease in that proportion as a result of inbreeding.

*Effect of long duration of the fertilized egg stage on the proportion of male-producers*

**DURATION OF THE FERTILIZED EGG STAGE.** Whereas parthenogenetic eggs hatch pretty uniformly in twelve to fourteen hours after laying, great variability has been found in the length of time which fertilized eggs from the same source, or from different sources, spend in the egg stage. Thus, in the cross between New York females and Baltimore males described in an earlier paper (Shull, '11 a), 408 eggs were obtained from 38 matings made from May 14 to May 17, inclusive. On May 24, three of these eggs hatched; and each day thereafter, with four exceptions, up to June 10, one or more eggs hatched. In the seventeen days from May 24 to June 10, 53 eggs, laid by 19 out of the 38 females, hatched. The remaining 355 eggs were kept two months longer, to August 10, but no more of them hatched, and the lot was then discarded. In no case did all the eggs laid by one female hatch, the highest record being six out of seven; while 19 of the females laid eggs none of which hatched within twelve weeks. It is probable that many of these would never have hatched.

About the same time, May 12 to May 15, 25 females of the Baltimore line were inbred, that is, paired with males of the same line.

They laid 298 eggs, none of which hatched before August 10, at which time they were discarded. A portion of this lot of eggs was kept cool (11° C.) for over seven weeks and then brought to room temperature; and another portion was frozen for thirty-three hours and then gradually thawed out. But none of these eggs hatched within twelve weeks, after which time observations ceased.

The F<sub>1</sub> females mentioned in the same paper (op. cit.) were inbred, but of 146 eggs laid, none hatched in seven weeks. Likewise, in the cross between F<sub>1</sub> and the Baltimore parent line, which were related lines, of 179 eggs none hatched in seven weeks. In the cross between F<sub>1</sub> and the New York parent line (also related lines), of 814 eggs, two hatched in about a week, while no more hatched in the next seven weeks.

In the above cases there is great variability in the duration of the egg stage in eggs coming from the same source; and there is considerable difference between eggs coming from one source and those from another source. Thus, crosses between unrelated lines yielded the greatest percentage of viable eggs; crosses between related lines yielded few or no viable eggs, while inbreeding failed to produce eggs that would hatch at all.

Since that time I have obtained numerous viable 'inbred' eggs, and crosses that produced a much higher percentage of viable eggs; but in none of these cases were complete records kept, hence comparative figures are not available.

It has already been demonstrated (Shull, '11 a) that fertilized eggs from different sources may yield parthenogenetic lines including different proportions of male-producers. The great variability in the duration of the egg stage in eggs from the same source, described above, suggested that this variability might be related to the proportion of male-producers in the parthenogenetic lines derived from those eggs; that is, that parthenogenetic lines derived from eggs that hatched quickly might include more or fewer male-producers than lines derived from late-hatching eggs. The following experiments were performed to test this possibility. The eggs used are the 'inbred' eggs obtained by pairing males and females of the same line (the 'original line' in Experiment 1).

*Experiment 3.* A fertilized egg which hatched in not less than 7 nor more than 14 days became the parent of the line in the first column of table 5, while the second column represents the line obtained from an egg that hatched in about four weeks.

The individual from the early-hatched egg yielded a line with 22.3 per cent of male-producers, the late-hatched only 15.4 per cent, notwithstanding that the 'early-hatched' line is the older line by two or three weeks and might therefore be expected to have decreased somewhat in the proportion of male-producers.

TABLE 5

*Showing number of male- and female-producers in two lines of Hydatina senta, one derived from a fertilized egg that hatched in two weeks or less after laying, the other from an egg that hatched in four weeks*

EARLY HATCH			LATE HATCH		
Date of first young	Number of ♂ ♀	Number of ♀ ♀	Date of first young	Number of ♂ ♀	Number of ♀ ♀
<i>November</i>			<i>November</i>		
22	1	11	22	2	11
24	4	10	24	4	17
26	1	7	26	4	28
28	9	25	28	6	37
30	0	41	30	1	24
<i>December</i>			<i>December</i>		
2	2	33	2	1	18
4	1	43	4	1	22
6	20	20	5	7	27
9	11	10	8	1	40
17	17	2	10	11	0
11	2	13	8	8	25
13	0	17	15	1	8*
15	3	14*			
Total.....	71	246		47	257
Per cent of					
♂ ♀ .....	22.3			15.4	

\* Remainder of family not recorded.

This possible decrease can hardly be urged in this case, however, as a reason for assuming that the effect of late hatching is even greater than table 5 shows it to be. For the 'early-hatched' line is here taken at a period of many male-producers. Prior to the opening of this experiment, the early hatched line included 16 generations. If these 16 generations be included with the 12 generations in table 5, the total for the early hatched line is 147 male-producers and 732 female-producers, or 16.7 per cent of male-producers. This is not greatly in excess of the 15.4 per cent in the late hatched line.

It may be pointed out incidentally that the average size of family in the late hatched line exclusive of the last family which was not fully recorded, is 26.8. In the early hatched line, if we count only the generations given in table 5, the average size of family is 25; but if the 16 earlier generations be included, the average size of family is 30.7. There is perhaps here a decrease of vigor associated with duration of the egg stage.

*Experiment 4.* Of the same lot of fertilized eggs as that used in the preceding experiment, one hatched after not less than 75 days nor more than 91 days in the egg stage, which is a much longer time than most of the other eggs of the same lot required. A line of 12 generations was bred from this individual, and is given in the right column of table 6. No line derived from an early hatching egg of this same lot was in existence at that time to compare with the late hatching line. The 'original line' (of Experiment 1) from which the lot of inbred eggs was obtained was still being reared, and that part of it which occurred simultaneously with the late hatched line in table 6 is given for comparison; but the original line is so much older than the late hatched line that we should expect it to have fewer male-producers even if it were at first equal to the late hatching line in the proportion of male-producers. This expectation is justified by the percentages in the table, where the original line gives only 9.4 per cent of male-producers, whereas the entire original line, including 39 generations previous to the seven here given, gave 17.3 per cent of male-producers. This is only a little less than the percentage obtained from the late hatching egg. It should also be recalled

TABLE 6

Showing the number of male- and female-producers in two lines of *Hydatina senta*, the one being reared from a resting egg that remained unhatched seventy-five to ninety-one days, the resting egg having been obtained from inbred female of the other line in this table.

ORIGINAL LINE			LATE HATCHING INBRED		
Date of first young	Number of ♂ ♀	Number of ♀ ♀	Date of first young	Number of ♂ ♀	Number of ♀ ♀
January			January		
9	1	7	9	2	14
	2	16	11	6	21
12	1	11	13	3	10
	1	6		0	8
14	2	2	15	0	21
	0	7	17	3	8
	0	2		1	10
	1	8	19	7	24
18	0	3	21	1	35
	0	4	23	2	24
21	0	2	25	0	21
30	0	5	26	12	22
	0	2	28	15	4
February				2	25
1	0	2	February		
			1	4	14
Total	8	77		58	261
Per cent of ♂ ♀		9.4			18.1

that the three other inbred lines recorded in the preceding experiments yielded 19.8 per cent, 16.7 per cent, and 15.4 per cent of male-producers respectively.

These percentages are so nearly equal that, on the whole, it seems pretty certain that there is no connection between the length of time spent in the resting egg, and the proportion of male-producers.

It may be pointed out incidentally that in the entire original line, including 38 generations previous to the seven recorded in table 6, but excluding those not recorded in full, the average size of family was 26.3, while the average size of family in the late

hatched line here shown is only 21.2. How much of this decreased vigor may be correlated with long duration of the resting egg stage, and how much with inbreeding, can not be ascertained from the experiment.

*Period at which the nature of a female is determined*

At what time in the life of an individual is it determined whether she will be a male- or a female-producer? Is it determined at a definite period, or is it a gradual process covering a long interval? These questions were answered variously by the early students of Hydatina. Nussbaum ('97) believed that a young female if treated properly could be made to produce either males or females, at the will of the experimenter. A young female that was starved, became according to his view, a male-producer. Maupas ('91), on the other hand, concluded that the nature of the female was determined in the egg from which she hatched, a conclusion which he expressed by saying that the sex of the offspring is determined in the body of the grandmother. Though Woltereck ('11) apparently accepts Maupas's view, partly because a female produces only males or only females, partly perhaps because of some provisional statements of my own (Shull, '10), statements which were confirmed in a paper subsequent to Woltereck's (Shull, '11 b), hitherto there has been no satisfactory proof of either proposition.

It has now been found possible to determine the time, or at least one time, at which the character of a female is decided, in a manner that seems to me conclusive. Both fertilized eggs and parthenogenetic eggs afford evidence on this point.

EVIDENCE FROM FERTILIZED EGGS. It has long been known that fertilized eggs of Hydatina produce only females. I find no statement, however, as to whether these females are always female-producers, or whether they may be of both kinds. They could not be all male-producers, or the species would have perished long ago.

In my breeding experiments I have reared to maturity 469 females from resting eggs, and every one has been a female-pro-

ducer. They were reared under the same conditions as other parthenogenetically produced females, many of which were male-producers. It is safe to conclude, therefore, that at the moment of fertilization it is determined not only that the immediate offspring shall be female, but that the individuals of the next generation shall be females. Whether this may be spoken of as sex determination a whole generation in advance, or not, is not clear; for no matter whether a male egg develops parthenogenetically and produces a male, or is fertilized and yields a female, the next generation in the direct line, if there be any, is necessarily always female. Aside, however, from the use of the word 'sex-determination' there can be no doubt that in this case the nature of the females of the first generation is determined in the fertilized eggs from which they hatch, and before those eggs are laid. There is no *a priori* reason, therefore, for supposing that the nature of other females may not also be determined in the parthenogenetic eggs from which they hatch, and before those eggs are laid.

EVIDENCE FROM PARTHENOGENETIC EGGS. The discovery that rotifers bred in a fairly strong solution of horse manure may be made to yield only female-producers, as pointed out in my earlier article (Shull, '10), was employed in the following experiments.

*Experiment 5.* A line of rotifers was reared in spring water. When the first members of a new generation were isolated, two were reserved for further breeding. One of these, together with all her offspring, was kept in spring water to continue the line. The other was reared to maturity in spring water, being examined every twelve hours, at 9 A.M. and 9 P.M., daily. As soon as she was observed to have laid eggs, she was transferred to a new dish of spring water, in which she remained during the next twelve hours; while the water on the eggs already laid was removed, and replaced with filtered, undiluted manure solution. Every twelve hours thereafter the female was transferred to fresh spring water, while the eggs laid in the preceding twelve-hour period were placed in manure solution. The eggs were allowed to hatch in manure solution and the young were reared to maturity in the same solution. Accordingly, every egg was laid in spring water, and (with

the exception of three eggs that hatched in less than twelve hours) was hatched, and the young reared to maturity in manure solution. That the manure solution used was strong enough to exclude male-producers was shown by rearing five successive generations in it. These five generations comprised 185 individuals, all female-producers, while the sister line in spring water, as shown in table 7, included many male-producers.

TABLE 7

*Showing number of male- and female-producers in two series of generations bred from sister individuals of Hydatina senta, in one of which the eggs were laid and the young reared to maturity in spring water, in the other the eggs were laid in spring water, but hatched and the young reared to maturity in manure solution.*

HATCHED AND REARED IN SPRING WATER			HATCHED AND REARED IN MANURE SOLUTION		
Date of first young	Number of ♂ ♀	Number of ♀ ♀	Date of first young	Number of ♂ ♀	Number of ♀ ♀
<i>February</i>			<i>February</i>		
8	25	15	8	9	38
9	19	30	9	38	9
11	8	31	11	13	26
12	18	31	12	7	33
Total.....	70	107		67	106
Per cent of					
♂ ♀.....		39.5			38.7

If the nature of a female is not determined before the twelfth hour of the egg stage, the treatment just described should exclude male-producers from the one line. If the nature of a female is determined at some time between the first and twelfth hours of the egg stage, the treatment described should cause a reduction in the proportion of male-producers in the one line; and the later the determination occurs, the greater should be that reduction.

From table 7 it appears that the young rotifers reared, from the egg stage on, in manure solution, comprise approximately as many male-producers as those reared in spring water. The fact that there is little or no reduction in the proportion of male-producers in manure solution seems to indicate that the nature of the female



is determined at, or prior to, an early egg stage; or if this determination is a gradual process, it has proceeded so far before the early egg stages that manure solution is unable to reverse it.

*Experiment 6.* In this experiment, a line was reared in spring water, and as in the preceding experiment two sister females were reserved from each generation for the purpose of breeding. One female was kept in spring water all her life, and all her offspring were reared to maturity in spring water. The other female was kept in spring water until she had laid 13 to 18 eggs, and was then transferred to filtered, undiluted manure solution. She was transferred to fresh manure solution every twelve hours thereafter, to prevent accumulation of bacteria, so that all of her eggs after the thirteenth to eighteenth were laid in manure solution. All the eggs laid in spring water were hatched, and the young reared to maturity, in spring water. All the eggs laid in manure solution were hatched, and the young reared to maturity, in manure solution.

That the manure solution used in this experiment was strong enough to exclude male-producers was shown by rearing in it five successive generations of all female-producers, as described in the preceding experiment. Further proof of its effectiveness is found in the additional control presently to be described.

The details of this part of the experiment are given in table 8, where the rotifers of the early part of the family, that were reared in spring water, are given to the left of the vertical line, those reared in manure solution to the right of that line. The offspring are recorded in the order in which they hatched, this order being determined fairly accurately, I believe, by the relative sizes of the young rotifers when they were isolated. Only one male-producer hatched from an egg laid in manure solution, and that came from the very first egg laid after the transfer of the female to the manure solution.

The control of this experiment, that is, those families bred entirely in spring water, is given in table 9. Each family was reared from a sister of the parent of the corresponding family in table 8. These control families are divided by the vertical

TABLE 8

Showing the number, and order of production, of male- and female-producers of *Hydatina senta*, in seven families, the early eggs of which were laid and hatched, and the young reared to maturity, in spring water, the later eggs laid and hatched, and the young reared in manure solution. Male-producers are designated by ♂, female-producers by ♀.

[illegible]

TABLE 9

Showing the number, and order of production, of male- and female-producers of *Hydrotia senta*, in seven families, all of which were reared to maturity in spring water. The parents are sisters of the parents in table 8. The vertical line divides each family at the point where, in the corresponding family in table 8, the female was transferred to manure solution. Male-producers are designated by ♂, female-producers by ♀.

[illegible]

line at the point where, in the corresponding family in table 8, the female was transferred to manure solution.

The two tables (8 and 9) together show in an unmistakable manner that male-producers have been quickly excluded from the latter part of the families in table 8 by transferring the parents to manure solution. Only one of the young produced in the manure solution was a male-producer, and that one hatched from the very first egg laid after the transfer. This one case is important as indicating that the nature of a female is determined prior to the laying of the egg from which she hatches; or if that determination is a gradual process, it has proceeded so far prior to the laying of the egg that manure solution is unable to reverse it.

*Experiment 7.* In this experiment a line was bred in manure solution. From each generation two sisters were reserved for breeding. One of these females was kept throughout life in manure solution, and all her offspring were reared to maturity in the same solution. The line thus reared consisted of 121 individuals, all female-producers, showing that the manure solution was strong enough to exclude male-producers from the families then being reared.

The other female, of the two reserved for breeding, was kept in manure solution until she had laid from 1 to 16 eggs, and was then transferred to spring water, where she produced the rest of her family. The eggs laid in manure solution were hatched, and the young reared to maturity, in manure solution. The eggs laid in spring water were hatched, and the young reared to maturity, in spring water. The details of this experiment are given in table 10. The vertical line divides each family at the point where the parent was transferred to spring water.

Many of the females hatched from eggs laid in spring water were male-producers, notwithstanding that their parents had previously been in manure solution strong enough to exclude male-producers. This indicates that the nature of a female is not determined in the very early (oögonial) stages of the egg from which she hatches. Of particular interest in this connection is the second family of table 10, from which it appears that the very first egg laid after the mother was transferred to spring water

yielded a male-producer. It is quite possible that an error was made in determining the relative ages of these first individuals, for, as stated above, the relative ages of the young rotifers isolated at one time was determined from their relative sizes. When the oldest rotifers were much alike it was sometimes difficult to determine relative ages. But in any case, I do not think it is possible that I have misplaced this individual by more than one step in order of age. That is, the first male-producer in the second family of table 10 can hardly have been later than the second young produced after the mother was transferred to spring water.

TABLE 10

*Showing number, and order of production, of male- and female-producers in seven families of Hydatina senta, in which the early eggs were laid and hatched, and the young reared to maturity, in manure solution, the later eggs laid and hatched, and the young reared to maturity, in spring water*

NUMBER OF FAMILY	LAID, HATCHED AND REARED IN MANURE SOLUTION	LAID, HATCHED, AND REARED IN SPRING WATER
1		♀ ♀ ♂ ♀ ♀ ♂ ♂ ♂ ♀ ♀ ♀ ♀ ♂ ♂ ♂
2	♀ ♀ ♀ ♀ ♀ ♀ ♀ ♀ ♀ ♀ ♀	♂ ♀ ♀ ♂ ♀ ♂ ♀ ♂ ♂ ♀ ♀ ♀ ♀ ♀ ♀ ♀
3	♀ ♀ ♀ ♀ ♀ ♀ ♀ ♀ ♀ ♀ ♀ ♀	♀ ♀ ♀ ♀ ♀ ♀ ♀ ♀ ♀ ♀ ♀ ♀
4		♀ ♀ ♀ ♀ ♀ ♀ ♀ ♀ ♀ ♀ ♀ ♀ ♀ ♀ ♀ ♀
5	♀ ♀ ♀ ♀ ♀ ♀ ♀ ♀ ♀ ♀ ♀ ♀ ♀	♀ ♀ ♀ ♀ ♂ ♀ ♂ ♀ ♀ ♀ ♀ ♀ ♀ ♂ ♂ ♀ ♀
6	♀ ♀ ♀ ♀ ♀ ♀ ♀ ♀ ♀ ♀ ♀ ♀ ♀ ♀ ♀ ♀ ♀	♀ ♀ ♀ ♀ ♀ ♀ ♀ ♀ ♀ ♀ ♀ ♀ ♀ ♀ ♀ ♀ ♀
7	♀ ♀ ♀ ♀ ♀ ♀ ♀ ♀ ♀ ♀ ♀ ♀ ♀ ♀ ♀ ♀ ♀	♀ ♀ ♀ ♀ ♀ ♀ ♀ ♀ ♀ ♀ ♀ ♀ ♀ ♀ ♀ ♀ ♀
		♂ ♂ ♂ ♀ ♀ ♂ ♀ ♂ ♂ ♂ ♂ ♂ ♂ ♂ ♂ ♀

The average number of eggs laid by these females on the days when they were transferred to spring water was 14.4 per day. That is, an interval of 1.66 hours elapsed between the laying of two successive eggs. Since the first male-producer in the second family in table 10 was not later than the second one produced after its mother was transferred to spring water, the nature of this female was not determined until within  $2 \times 1.66$  hours, or 3.32 hours, before laying.

Experiments 6 and 7 together indicate that the nature of a female (with respect to the kind of offspring she will produce) is determined before the egg from which she hatches is laid, but not until within several hours of the time when the egg is laid. Or, if this determination is a gradual process, it has proceeded so far before the egg is laid that manure solution can not reverse it, but has not proceeded so far until within several hours of laying, but that manure solution can reverse it. Microscopic examination of the living animals, which are so transparent that the eggs and oögonia may readily be seen, shows that the last several hours of the egg stage, within the parent's body, includes the entire growth period.

#### DISCUSSION

The decrease in the proportion of male-producers with long-continued parthenogenesis, which was shown to occur in some parthenogenetic lines of *Hydatina*, is of interest from several points of view. First, may not this decrease account for part of the differences observed between parthenogenetic lines in cases where the ages of the lines are not known? If one line, started immediately from a fertilized egg, be compared with another removed by a hundred generations from the fertilized egg, the latter line might be expected to show fewer male-producers, even if in their early generations both lines had been equal.

If differences between parthenogenetic lines may thus be secondarily produced, how does this phenomenon affect the results of crossing reported in my earlier paper (Shull, '11 a)? That depends on the relative ages of the lines. The Baltimore line was started from a female collected in March. The winters are sufficiently rigorous in Baltimore, I think, to prevent continued reproduction during that season. A female collected in spring, therefore, must descend from a fertilized egg that hatched probably not earlier than February of the same year. The Baltimore line can hardly have been more than a month or two old when I obtained it. Regarding the age of the New York line there is less certainty. The parent of this line was found in January in a culture in the laboratory, which had been stocked with

rotifers more than two years before, and to which none had been added since. This culture had been examined many times for rotifers, but none were seen until the single specimen which produced the line recorded as the New York line was found. I am inclined to think, therefore, that this female had recently hatched from a fertilized egg, and that the New York line was accordingly about a month older than the Baltimore line. Whether this difference in age may account for the difference in the proportion of male-producers between the two lines is uncertain.

That differences in the proportion of male-producers not dependent on differences in age may exist between two lines is shown, however, by another experiment, in which the  $F_1$  line was crossed back to the New York line, and in several other cases not recorded in that paper. In the cases to which I refer, the older line produced more male-producers than the younger line.

Uncertainty as to the age of the original lines, therefore, can not invalidate the conclusion that differences dependent on an internal agent do exist between parthenogenetic lines; it merely modifies our conception of the nature of those differences, a subject that is discussed elsewhere.

Some of the long parthenogenetic lines recorded in table I, it is to be noted, do not show an evident decrease in the proportion of male-producers; nor do they show an increase. Each of these lines began with a low percentage of male-producers, and could not have decreased much. These lines showed considerable fluctuations in the proportion of male-producers, periods of few male-producers being followed by periods of many. If such a fluctuating line began with a long period of few male-producers, a decrease in the proportion of male-producers could only be discovered by breeding it through a large number of generations, including several waves of male-producers, and finding that successive waves were less marked. Forty-six generations are hardly enough for this. The progressive decrease in the proportion of male-producers is so marked in some cases, and its absence in some lines so easily explained, that I am inclined to regard it as a general phenomenon.

Just such a progressive change occurs in daphnians (Woltereck, '11), but here the number of sexual individuals increases with the age of the line,<sup>3</sup> instead of decreasing as in *Hydatina*. That the change should be in the opposite direction in the rotifers and daphnians need hardly surprise one, since other phenomena are reversed in the two groups. For example, late females in a family of daphnians produce more sexual daughters than do their older sisters, while late females in a family of *Hydatina* produce fewer male-producers (sexual females) than do the early females.

The progressive decrease in the proportion of male-producers may also have a bearing on pure line<sup>3</sup> work in general. So far as we know there is no method by which parthenogenesis may change the genotypic constitution of a line, yet parthenogenetic lines of *Hydatina* do change. May not pure lines suffer progressive change, notwithstanding they are composed of homozygous individuals? If so, there can be differences between two pure lines having the same genotypic constitution, even when both are reared under the same external conditions. If this were found to be true, it would not invalidate the conclusion that pure line differences exist, but would modify our explanation of them and their apparent behavior in inheritance.

How this progressive decrease in the proportion of male-producers is brought about is not known. At first, it seemed that the proportion of male-producers might be determined by the vigor of the parthenogenetic line. The view that long-continued reproduction, whether bi-sexual, parthenogenetic, or vegetative, without the introduction of new 'blood' in crosses, is detrimental to vigor, is often expressed, even if not always correct. Such a loss of vigor seems to occur in *Hydatina*, as evidenced by the decrease in the size of family in successive parthenogenetic gen-

<sup>3</sup> In an earlier paper I have spoken of a series of parthenogenetic generations in *Hydatina senta* as a pure line. While parthenogenetic species do not meet the requirement of Professor Johaussen's definition of a pure line, there seems to be no abuse of the fundamental conception of pure lines in applying the term to parthenogenetic species. The term klon, or clone, used by plant geneticists to denote vegetatively produced varieties, can hardly be used for *Hydatina*, since there is a wide step between parthenogenesis and vegetative reproduction. Under these circumstances I have preferred to use the term parthenogenetic line in the present paper.

erations (figs. 5 and 6). The simultaneous decrease of vigor and of the proportion of male-producers could be readily 'explained' in part by assuming that the two phenomena are correlated.

Opposed to this view is the fact that inbreeding in *Hydatina* seems on the whole, to result in a diminution of vigor, whereas the proportion of male-producers appears on the average to be unaltered by inbreeding. Such could not be the case if any true correlation between the two phenomena existed. A further objection to the view that vigor and the proportion of male-producers are correlated is found in the results of experiments (3 and 4) in which lines derived from females that remained long in the fertilized egg are compared with lines derived from females that hatched quickly from the egg. If the duration of the egg stage is inversely proportional to vigor, as one might expect, a correlation between vigor and the proportion of male-producers would result in a lower percentage of male-producers in parthenogenetic lines derived from late hatching females. This appears not to be the case. The decrease in vigor due to inbreeding may be accounted for if we assume that vigor is due to the degree of heterozygosis of the individuals, as has been found to be the case with corn (G. H. Shull, '08). But this assumption will not explain the decrease of vigor with long-continued parthenogenesis (figs. 5 and 6).

We have seen that the internal nature of a parthenogenetic line of *Hydatina* is subject to some degree of change dependent on the age of the line, but that initial differences also exist among different lines. We may thus conceive the internal nature, as far as it concerns the life cycle, to be composed of at least two parts: First, the genotypic constitution, determined at the moment of fertilization, and, barring irregularities partaking of the nature of mutations, remaining constant through many generations; and second, a changeable element which is probably to be included in Woltereck's reaction-norm. We may either add to these a third element which causes the great fluctuations ('waves') in the proportion of male-producers, or assume that the reaction-norm is itself very variable.



Of the second factor, little can be said except in a descriptive way. In *Hydatina* it progressively changes so that the proportion of male-producers decreases with the age of the parthenogenetic line. Whether this change is due to continued breeding under uniform conditions, or to some other cause, is not known. Little more can be said of the variable element, whether separate from or only a feature of the progressive one. Fluctuations in the 'sexuality' of daphnians occur, such that periods of few sexual forms may alternate with periods in which sexual individuals are numerous. Woltereck ('11) attributes the form of the cycle to antagonistic substances, now the one, now the other gaining the ascendancy in a rhythmical manner. I have found in *Hydatina* just such fluctuations, which I have not been able to trace to any external agent. Nevertheless, it appears that the extent of the fluctuations is not independent of external conditions. Thus, in my earlier starvation experiments (Shull, '10, fig. 1), both the starved and the well-fed lines show simultaneous fluctuations in the same direction, but in every case the wave is more marked in the well-fed line than in the starved. Even if the external conditions (chemical substances in the water, for example) are not the cause of this fluctuation, they do modify its amplitude.

Regarding the first element of the internal nature of *Hydatina*, the genotypic constitution (zygotic constitution of Punnett, '06), we fortunately have more evidence. The crossing experiments described in my former paper (Shull, '11 a), together with the results of inbreeding described in this article, enable us at least to eliminate certain possible views regarding the internal cause of the form of the life cycle.

The proportion of male-producers can not be dependent on the simple quantity of some substance present. For it is difficult to see why, in some crosses, the  $F_1$  line should be intermediate in the proportion of male-producers between its parent lines, while in other crosses the proportion in  $F_1$  should exceed not only that of either parent line alone, but of both parent lines combined (op. cit., Experiments 36 and 35).

Among Mendelian explanations, it can not be assumed that the life cycle in a given line is dependent on a single gene or a pair of

genes, representing a certain proportion of male-producers. For this explanation could not account for intermediate  $F_1$  in some cases, and an  $F_1$  higher than both parent lines in other cases.

If we assume that many genes participate in the production of the cycle, many of the results so far obtained are easily explained. If we think of these genes for male-producers as being all alike, equipotent, and additive in their effects, so that six genes produce twice as many male-producers as three genes; we should then have to assume, in order to explain the crosses described in my former paper, that the percentage of male-producers is proportional to the number of genes for which the line is heterozygous. This explanation seemed plausible when it was thought that vigor and the life cycle were correlated; for in corn it seems probable that vigor is dependent on the degree of heterozygosis. The crux of this explanation is found in the results of inbreeding. If the percentage of male-producers is proportional to the number of genes for which the line in question is heterozygous, inbreeding, by reducing the number of genes for which the line is heterozygous, should rapidly reduce the proportion of male-producers. This it does not do. Inbreeding results in a line that includes practically the same proportion of male-producers as the line from which it was derived. Even twice inbreeding, or inbreeding a line itself the result of inbreeding, does not certainly show a reduction in the percentage of male-producers.

It can not be assumed, therefore, that the genes for the proportion of male-producers are all alike and effective in proportion to their numbers. Instead, we may assume that the life cycle is dependent on a number of genes *not* all alike, some being more effective than others, and some combinations producing more male-producers than other combinations, even when these combinations involve the same number of genes. That something akin to segregation of these representatives of the cycle occurs, is made probable by the fact that crosses between the same parthenogenetic lines are not equal with respect to the proportion of male-producers. That the genes are not alike nor additive in their effects is shown by the fact that a cross may result in a higher proportion of male-producers than in both parent lines combined.

The effect of crossing on the cycle can not be predicted, therefore, from the form of the cycle in the lines to be crossed, but only after tests are made by experiment.

Whatever be the nature of the genotypic constitution, the form of the cycle in a parthenogenetic line having a given constitution is dependent in part upon the environment. It was earlier shown that certain chemical substances were capable of reducing the proportion of male-producers. From evidence presented in this paper, we may now conclude that the effect of these substances is felt only during the growth period of the egg. Once the egg has reached its full growth, or at least after it has been laid, chemical substances which, when applied throughout life, exclude male-producers are powerless to change the nature of the female hatching from the egg. In like manner, these substances are powerless to affect the nature of a female before the egg from which she hatches begins its growth. So far as these chemical substances are concerned, the fate of an egg is irrevocably determined in its growth period. Since the maturation spindle is formed in these eggs before they are laid (Whitney, '09), it is not impossible that the influence of external agents is limited to the maturation period.

This localization of 'sex-determination' in the growth period is of interest in several connections. First, it shows why the starvation experiments of Punnett ('06) and Whitney ('07) did not result in an increased proportion of male-producers, as did the experiments of Nussbaum ('97) and myself (Shull, '10). Even if starvation, as carried out by the former two investigators, so altered the chemical composition of the water that a change in the life cycle might have been expected, nevertheless it was not applied at a time when it might have been effective. In the experiments of Punnett and Whitney, the females were starved only during the first few hours after hatching, not when their eggs were in their growth period.

The localization of the period susceptible to external agents also goes to disprove my former explanation of the observed fact that late daughters of a family yielded fewer male-producers than did their sisters of the early part of the family. I assumed that

the accumulation of certain chemical substances in the cultures as these became old might cause the offspring of the late females to be more largely female-producers than the offspring of early females. In the light of the discovery that the period susceptible at least to certain chemical substances is limited to the growth period, my former explanation regarding the later females of a family would account for a preponderance of female-producers in the last part of the family, but not for a preponderance of females that produce female-producers. A preponderance of female-producers in the last part of the family, as compared with the early part, does not occur, as was shown by compiling data from 349 families (Shull, '10).

A comparison with the Cladocera with respect to the susceptible period will be of interest. The Cladocera do not lend themselves to an inquiry of this kind as readily as do the rotifers, for the offspring of a daphnian are not all of one sex. However, according to Woltereck ('11), *Daphnia* has two 'labile' periods, one just before the eggs enter the brood chamber, the other very much earlier, in the oögonial stages. It seems not improbable that the labile period immediately prior to the entrance of the eggs into the brood chamber falls within the growth period, as in *Hydatina*.

And finally, not the least valuable result of the discovery that manure solution is effective only in the growth period of the egg, is that a way now seems open to discover the manner in which chemical substances affect the life cycle. The question whether these substances alter the events in a given cell, or whether they merely decide which of two already differentiated classes of cells shall develop, bids fair to be answered. If there are two classes of cells already differentiated, and manure solution prevents one of them from developing; and if eggs may come to the growth period before being affected by manure solution; then females from a line producing many male-producers, if placed in manure solution, should frequently show traces of degenerating eggs, or of eggs that do not develop and must be pushed aside to make room for cells of the other class. Observations on this point are now in progress.

## SUMMARY

A progressive decrease in the proportion of male-producers with long-continued parthenogenesis occurs in some lines of *Hydatina*, perhaps in all. It is not improbable that differences between parthenogenetic lines may thus secondarily arise, which are independent of both genotypic constitution and the immediate external environment.

A progressive decrease in the size of family with long-continued parthenogenesis occurs in some lines. There is apparently no correlation between decrease in size of family (decrease of vigor) and decrease in proportion of male-producers.

The time required by fertilized eggs to hatch varies from a few days to many weeks.

The length of time required for a fertilized egg to hatch is probably not correlated with the proportion of male-producers in the parthenogenetic line derived from the egg.

Parthenogenetic lines derived from fertilized eggs that require a long time to hatch may be less vigorous (as measured by size of family) than those from early hatching eggs.

Individuals hatching from fertilized eggs are not only all females, as previously known, but are all female-producers.

Whether a female is to be a male-producer or a female-producer is irrevocably decided (so far as manure solution is concerned) in the growth period of the parthenogenetic egg from which the female hatches.

Sex is determined a generation in advance.

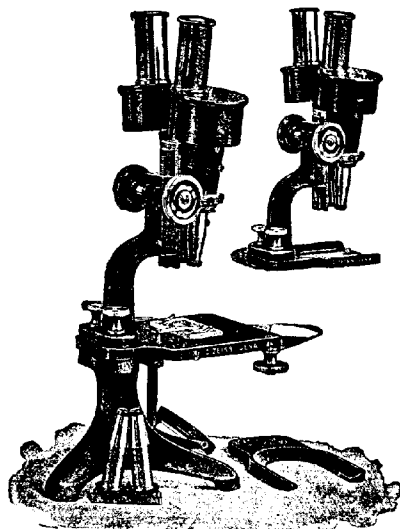
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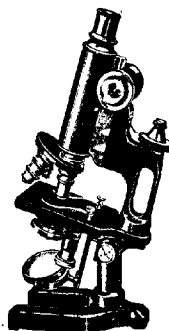
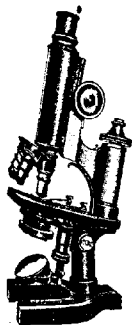
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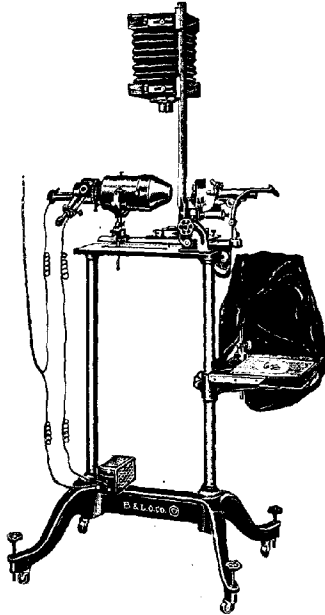
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## STUDIES ON SEX-DETERMINATION IN AMPHIBIANS

### V. THE EFFECTS OF CHANGING THE WATER CONTENT OF THE EGG, AT OR BEFORE THE TIME OF FERTILIZATION, ON THE SEX RATIO OF BUFO LENTIGINOSUS

HELEN DEAN KING

*From The Wistar Institute of Anatomy and Biology*

The investigations recorded in the present paper are a continuation of those that have been carried on for several years past in an attempt to ascertain whether external factors can influence the determination of sex in the toad, *Bufo lentiginosus*.

All of the experiments were made with the eggs from two females, *a* and *b*; the eggs from each female being fertilized with sperm from the same male. The individuals derived from the eggs of female *a* are considered to belong to the 'series A' group of experiments; while 'series B' refers collectively to the individuals that developed from the eggs of female *b*. It was not possible to note the exact number of eggs used in any experiment, but an attempt was made to use approximately the same number of eggs in each case, and to estimate, as accurately as possible, the number of eggs that failed to develop.

The apparatus that was used in rearing the tadpoles was described in detail in a previous paper (King, '11). As this apparatus has its limits of capacity, it was not possible to use all of the embryos that developed from each lot of eggs. Definite numbers of individuals, forming in every case except the acid experiments at least 75 per cent of the total number of eggs that had been experimented upon, were taken at random as they emerged from their jelly like membrane three days after the experiments were begun. In the various tables in this paper the figures given in the column headed 'total number of individuals' refer, there-

fore, to the number of tadpoles taken for rearing, and not to the number of eggs that had been used in making the experiment.

The tadpoles lived chiefly on *spirogyra*, *nitella*, and various other water plants taken from ponds in which toads normally breed each year. Occasionally they were fed on finely ground fish or frogs' muscle; but food of this kind that was not eaten within two or three hours was removed, as it fouled the water very quickly and so increased the mortality. Experience has shown that water plants, with their accompanying hordes of micro-organisms, form a food supply for toad tadpoles much superior to that used in any former experiments. (King, '07 b, '09, '10, '11).

In *Bufo* the sexes cannot be distinguished until the tadpoles are approaching metamorphosis, and even when young toads live until their tails have been absorbed it is necessary to section the gonads in a considerable number of cases in order to ascertain the sex. Several attempts were made in past years to feed young toads so that they might live until their gonads were well differentiated; but such attempts were failures, owing to the difficulty of obtaining a sufficient supply of small insects to feed a large number of individuals. Last spring it was found that young toads would eat various species of aphids and grow rapidly on a diet composed chiefly of these insects. As this food could be obtained in considerable abundance, nearly all of the individuals used in the various series of experiments were kept alive for about three weeks after they had completed their metamorphosis. By this time the sex glands were so well differentiated that the sex could readily be ascertained by examining the gonads in toto under a dissecting lens.

The period of metamorphosis seems to be a critical one in the life history of toads reared under artificial conditions, as there is always an increased mortality at this time. All of the individuals that died at this stage of development were preserved in Tellyesnicky's fluid, which has been found far superior to corrosive-acetic as a fixative for the gonads of *Bufo*, and their sex ascertained by means of sections.

Altogether 4224 tadpoles were used in the various experiments, and of this number 3784 individuals were carried through to metamorphosis and their sex ascertained. The mortality in the entire series was, therefore, only 10.41 per cent, which is much less than the mortality that must occur in any lot of eggs laid under natural conditions. Whatever explanation may be offered for the unusual sex ratios obtained in some of these experiments it is evident that they cannot be ascribed to selective mortality.

According to Davenport ('97), water forms from 60 per cent to 90 per cent of the whole mass of protoplasm in nearly all kinds of cells and is of the utmost importance for the various chemical processes taking place in the living organism. It is conceivable, therefore, that placing eggs under conditions that would alter their water content just before or during the time of fertilization might favor the development of one sex or the other, if it be that the sex of an individual depends upon some definite metabolic process occurring during the fertilization period. Some investigations made along this line last year gave such suggestive results that this past spring I confined my experiments with the eggs of *Bufo* to an attempt to ascertain whether the normal proportion of the sexes would be altered if the water content of the eggs was changed at or before fertilization.

For convenience in description these experiments are divided into two classes: (1) those in which the water content of the unfertilized eggs was affected; (2) those in which the eggs were subjected to conditions that altered their water content during the fertilization period.

#### 1. EXPERIMENTS ON THE UNFERTILIZED EGG

Former experiments in which unfertilized eggs of *Bufo* were subjected to the action of hypertonic solutions of salt and of sugar gave results which strongly suggested that the normal sex ratio had been altered by the treatment which the eggs had received previous to their fertilization (King, '11). Unfortunately the mortality at the time the eggs were fertilized, and also among the tadpoles during the early stages of their development, was



very great. One could not exclude the possibility, therefore, that selective mortality was responsible for the results, even if there is no evidence that mortality is ever selective in amphibian tadpoles reared under artificial conditions.

In making the experiments mentioned above it was found that solutions of salt and of sugar as strong as  $2\frac{1}{2}$  per cent could not be used on the unfertilized eggs for more than five minutes without rendering the great majority of them incapable of being fertilized. In continuing these experiments very weak solutions were employed so that the mortality at the time of fertilization might be decreased.

A batch of about 400 eggs, taken from female *a*, was placed in a 2 per cent solution of cane sugar; another batch of approximately the same number of eggs was put in a 2 per cent solution of NaCl. Each lot of eggs remained in the solution for ten minutes, and was then quickly washed off in running water and fertilized in tap water. At least 95 per cent of the eggs that had been subjected to the action of the salt solution segmented normally. Comparatively few of the tadpoles died during the early stages of development, and the entire number of individuals in which sex was not ascertained was only 12 per cent. The results obtained with the eggs that had been placed in the sugar solution were even more satisfactory. Not more than 2 per cent of the eggs failed to develop, and only 7 per cent of the 300 individuals that were taken for rearing died before it was possible to ascertain their sex. In each of these lots, as shown in table 1, a percentage of females was obtained that was considerably higher than that found among the toads that served as control for the experiments in this series. The latter individuals were developed from eggs of female *a* which had been fertilized in tap water with sperm from the same male that was used in the fertilization of all of the other eggs taken from this female.

Eggs, taken from female *b*, were subjected to the action of a 2 per cent solution of sugar for twenty minutes and were then fertilized in tap water; only about 5 per cent of these eggs failed to segment. In 53, or 22.40 per cent, of the 250 tadpoles that were taken for development, the sex was not ascertained. This

loss was due, in great part, to an accidental contamination of the water in two of the dishes containing the tadpoles and not to the treatment that the eggs received at the time that they were ready for fertilization. The lot of toads carried through metamorphosis gave a percentage of females nearly 10 points above that in the control for the series, and slightly greater than that found among the individuals belonging to the corresponding experiment in series A (table 1).

Owing to the fact that solutions of NaCl are much more injurious to the eggs of *Bufo* than are sugar solutions, the experiment in which eggs from female *b* were immersed for twenty

TABLE 1  
*Eggs treated with hypertonic solutions before fertilization*

SERIES	SOLUTION USED	TIME OF ACTION min.	TOTAL NUMBER OF INDIVIDUALS	NUMBER SEX AS CERTAINED	MALES	FEMALES	PER CENT F ♀ MALES	NUMBER MALES TO 100 FEMALES
A	2 per cent sugar	10	300	279	131	148	53.04	88.51
A	2 per cent salt	10	200	176	70	106	60.34	66.03
B	2 per cent sugar	20	250	194	86	108	55.66	79.62
A	Control		350	322	169	153	47.50	110.45
B	Control		350	334	178	156	46.40	114.10

minutes in a 2 per cent solution of NaCl before fertilization was a failure. Only a few of the eggs segmented, and as all but twelve of the embryos died during gastrulation the experiment had to be abandoned.

In each of the experiments outlined above there was found among the individuals carried through to metamorphosis a percentage of females considerably above that in the control lot. These results accord well with those obtained in former experiments with hypertonic solutions (King, '11), although the percentages of females are somewhat lower, owing possibly to the fact that the eggs were treated with weaker solutions.

Taking the sex ratio for any lot of individuals as the number of males to each 100 females, it is found that the toads derived from eggs that were subjected to the action of the salt solution before fertilization give a much lower sex ratio than that occurring among the individuals developed from eggs which had been treated with sugar solution (table 1). If this difference can be attributed to the fact that the osmotic action of salt is several times greater than that of sugar, it follows that the more water that is extracted from the egg just before fertilization the greater becomes its tendency to produce a female rather than a male. On this assumption it is the egg, and not the sperm, that contains the sex-determining mechanism. In *Bufo*, therefore, as in the sea-urchins according to the recent investigations of Baltzer ('09), the female is heterozygous as regards sex and the male is homozygous.

The above interpretation of these results is not the only one that can be given, although it seems to me to be the most plausible. Selective mortality cannot be held responsible for the sex ratios obtained, since in none of the experiments was the mortality sufficiently great, either at the time that the eggs were fertilized or during the development of the tadpoles, to have appreciably affected the results. There are two possible explanations for these results that do not involve the admission that external factors can influence sex. It is conceivable that subjecting eggs to the action of hypertonic solution just before their fertilization may have rendered them more easily penetrated by spermatozoa that were female-producing than by those that were male-producing, assuming that the spermatozoan determines sex as the current chromosome sex theory demands. This means, however, that fertilization must here be considered as selective, though Wilson ('10) has recently shown that selective fertilization is most improbable in any form. A study of the spermatogenesis of *Bufo* (King, '07) has not shown any dimorphism of the spermatozoa that might be associated with sex-determination; neither has such a dimorphism been found in the spermatozoa of any amphibian so far investigated. It seems somewhat absurd, therefore, to assume the existence of dimorphic spermatozoa in *Bufo*.

in order that the result of these experiments may be ascribed to selective fertilization.

There remains the possibility that the sex ratios in these lots of individuals were chance variations in the normal sex ratio, and that the treatment to which the eggs were subjected, previous to their fertilization, had nothing whatever to do with the sex of the future embryos. In table 2 is given a summary of the proportion of the sexes and of the sex ratios in various lots of individuals that have served as controls for different series of experiments made during the past six years. The 500 young toads examined in 1904 were obtained from the banks of the Susquehanna River at Owego, New York, shortly after they had completed their metamorphosis under natural conditions. All of the other individuals used in computing the table were developed from the eggs of females obtained in the vicinity of Philadelphia, Pa. In every case the eggs were normally or artificially fertilized in laboratory tap water, and the tadpoles reared under very uniform external conditions. No lots of individuals have been included which developed from eggs that were subjected to any abnormal treatment, at or before the time of fertilization,

TABLE 2

*Sex ratios in various control lots of individuals*

YEAR	TOTAL NUMBER OF INDIVIDUALS	NUMBER SEX ASCERTAINED	MALES	FEMALES	PER CENT FEMALES	NUMBER MALES TO 100 FEMALES
1904		500	241	259	51.80	93.05
1907		600	259	341	56.83	75.92
		651	292	359	55.14	81.33
1908		140	64	76	54.28	84.21
1909		323	157	166	51.39	94.56
	210	134	65	69	51.41	94.20
	1250	775	372	403	52.00	92.25
1910	201	140	65	75	53.57	86.66
		200	94	106	53.00	88.67
1911	350	322	169	153	47.50	110.45
	350	334	178	156	46.40	114.10
Total...		4119	1936	2163	52.51	90.42

or in which the tadpoles were exposed to unusual conditions of temperature or of nutrition during the course of their development, although in many such cases the sex ratios obtained were very similar to those of control lots.

In the various lots of individuals whose sex data are included in table 2 the number of males to each 100 females varies from 75.92 to 114.10; when the percentages of females are compared there is found to be a difference of 10.43 points between the extremes of the series. These figures indicate that normally there is but little variation in the proportion of the sexes in different lots of toads. Table 2 shows also that there is no marked seasonal variation in the sex ratio of *Bufo*, such as Pffüger ('82) and von Griesheim ('81) claim is the case with frogs. The latter investigators based their conclusions on the sex ratios in adult frogs collected from different localities in different years. My investigations have been confined entirely to the sex ratios in young toads that have recently completed their metamorphosis. Judging from the proportion of the sexes in several hundred adult toads that I have collected at various times during the past ten years, the sex ratio in adult individuals is very different from that in the young, since among adults there appears to be a considerable excess of males which is particularly noticeable during the breeding season.

The sex ratios in the two lots of individuals derived from eggs that were subjected to the action of sugar solution before fertilization fall within the limits of normal variation in the sex ratio (table 2). There is, therefore, some ground for an assumption that these cases afford no evidence that the normal proportion of the sexes was altered by the treatment which the unfertilized eggs received. The sex ratio found in the individuals that developed from eggs that were treated with salt solution is considerably lower than that in any control lot so far examined; but this may, perhaps, be considered as an exceptional variation. If these sex ratios are mere chance deviations from the normal, it certainly seems very remarkable that all three of them should show such a high percentage of females.

## 2. EXPERIMENTS ON THE FERTILIZED EGG

If the sex of an embryo is not definitely fixed by the character of the spermatozoan that fertilizes the egg, it is possible that the zygote is a sex-hybrid and that external conditions, acting during the early stages of development, may turn the balance in favor of one sex or the other.

Several different experiments were made this year to see whether changing the water content of the zygote would have any effect on the sex ratio. These experiments may be divided into two groups: (A) those in which an attempt was made to cause the eggs to absorb an increased amount of water during the fertilization period; (B) those in which eggs were made to lose water during this time.

*With increased absorption of water*

According to Loeb ('06), eggs can be made to take up water by placing them in weak solutions of acid or of alkali, the quantity of water absorbed depending on the strength of the solution used. Former experiments have shown that the eggs of *Bufo* are very sensitive to the action of acid and of alkaline solutions, and that it is not possible to subject them to the action of a solution stronger than 0.01 per cent without rendering the great majority incapable of development. Last year seven lots of eggs, from four different females, were fertilized in weak solutions of acetic acid (0.0025 per cent to 0.01 per cent), and in every instance the percentage of females obtained was from 10 per cent to 20 per cent lower than that in the control lot. Unfortunately no definite conclusions could be drawn from these experiments, since in every case the mortality was very great both at the time that the eggs were fertilized and during the growth of the tadpoles.

I planned to repeat these experiments on a large scale this past spring in the hope that definite conclusions would be possible from the results obtained. To my great surprise, however, I found that it was not possible to obtain any considerable number of eggs that would develop normally after being fertilized in solutions of acetic acid. Altogether twenty batches of eggs, from

five different females, were experimented upon, and in no case did more than one-tenth of the eggs segment even when the solution used had a strength of only 0.0025 per cent. The failure of these experiments cannot be due to the chance selection of a particularly bad lot of eggs and sperm, since eggs from two of the five females were used for all of the other experiments that were made, and the very great majority of them developed normally although they were fertilized under very unusual conditions.

The only explanation that I can offer for this very unexpected result is that, when fertilization was attempted, the eggs happened to be in a physiological condition that rendered them particularly sensitive to the action of acid solutions. This past spring no toads were obtained until the seventh of April, and each of the five females used for these experiments had already laid a portion of her eggs before she was brought into the laboratory; the eggs were, therefore, very ripe. In 1910, females were obtained the latter part of March, and as none of them had laid any of their eggs when captured, the eggs were presumably in an early stage of ripening when they were experimented upon. According to Hertwig ('06), the physiological condition of amphibian eggs varies considerably at different phases of their ripening, and it may be, therefore, that very ripe eggs are more easily injured by acid solutions than are eggs that are in an earlier stage of development.

The individuals belonging to only one of the acid series were saved. In each experiment in the series about 400 eggs, taken from female *b*, were fertilized in solutions of acetic acid and removed to fresh water at the end of one-half hour. The strengths of solutions used, which were the same as those employed last year, are shown in table 3. Many eggs that segmented in a more

TABLE 3  
*Eggs fertilized in solutions of acetic acid*

STRENGTHS OF SOLUTIONS USED	TOTAL NUMBER OF INDIVIDUALS	NUMBER SEX ASCERTAINED	MALES	FEMALES	PER CENT FEMALES	NUMBER MALES TO 100 FEMALES
<i>per cent</i>						
0.01	46	42	22	20	47.62	110.00
0.0050	51	42	26	16	38.19	162.50
0.0025	27	19	14	5	26.31	280.00

or less normal manner died during the gastrulation period, so the number of tadpoles that could be taken for rearing was very small. The sex data obtained in this series are shown in table 3.

No conclusions can be drawn from these results since there were so few individuals in the various lots. The experiments have been recorded simply because the sex ratios found agree with those obtained in similar experiments made last year. Altogether ten different experiments have been made in which various lots of eggs have been fertilized in acid solutions, and in each case a very low percentage of females has been obtained. Such a consistent series of results, in so many different cases, strongly suggests that the acid solutions have increased the tendency of the eggs to produce males rather than females, presumably by causing them to absorb an increased amount of water during the fertilization period. In all of these experiments, however, the mortality was very great, so it is possible that selective mortality was responsible for the results; though why acid solutions should invariably be more injurious to young females than to young males is not at all clear. It will be necessary to repeat these experiments on eggs that are in a physiological condition to withstand the injurious action of acid solutions before any definite conclusions are possible regarding the effects of such solutions on the sex ratio of *Bufo*.

In another experiment eggs were fertilized in water that had been distilled in glass, in the hope that the zygote would absorb an increased amount of water and thus tend to produce a male rather than a female. The eggs, which were taken from female *a*, remained in the distilled water for thirty hours, the water being changed three times during this period. Practically all of the eggs experimented upon segmented in a normal manner and continued their development. Not many of the 400 tadpoles taken for rearing died during their early development, and the entire loss was only 13.75 per cent. The 345 individuals that were carried through to metamorphosis were found to consist of 189 males and 156, or 45.21 per cent of females. In this instance the sex ratio of 121.15 males to 100 females differs so little from that in the control for the series (table 1) that evidently the normal proportion of the sexes was not appreciably altered by the



conditions to which the eggs were subjected during the early stages of their development.

The results obtained in this experiment might seem to indicate that increasing the amount of water in the egg at the time of fertilization has no influence whatever on the process of sex-determination; but there is another possible interpretation of them which seems worth considering. The ripe eggs of the toad are surrounded by two membranes and embedded in a thick, jelly like substance. It is therefore possible that when eggs are fertilized in distilled water the osmotic pressure on them is, for some little time, practically the same as that to which eggs are subjected when they are fertilized under natural conditions. If this be so, the results of this experiment give no evidence whatever regarding the effects on the sex ratio of increasing the water content of the eggs during the period of fertilization.

Although the sex-determining mechanism was not affected by the distilled water, some change was produced in the eggs which had a decided influence on their later development. The tadpoles belonging to this lot were very small, and their development, although apparently normal, was so retarded that they were the last of all of the individuals in the various series of experiments to undergo metamorphosis.

None of the experiments in which an attempt was made to increase the water content of the zygote have given results that could be considered as conclusive. It is suggestive, perhaps, that in every instance a relatively low percentage of females has been obtained; but other methods of experimentation will have to be employed before it will be possible to determine whether increasing the amount of water in the eggs at the time of fertilization really leads to an alteration of the sex ratio.

#### *With loss of water*

It would seem to be an easy matter to reduce the water content of the zygote by fertilizing the eggs in a hypertonic solution and allowing them to remain in the solution for a considerable length of time. Unfortunately the spermatozoa of *Bufo* are very easily injured, and even 1 per cent solutions of salt or of sugar render the great majority of them incapable of fertilizing the eggs. In

continuing experiments of this kind it was considered necessary, therefore, to use very weak solutions in order that the mortality among the spermatozoa might be greatly reduced.

One batch of about 400 eggs, taken from female *a*, was placed with spermatic fluid in a  $\frac{1}{2}$  per cent solution of cane sugar; another batch of eggs from the same female was fertilized in a  $\frac{1}{2}$  per cent solution of NaCl. Each lot of eggs remained in the solution for one-half hour and was then transferred into fresh water. The mortality at the time of fertilization was slightly greater where salt solution was used, but in this case it was not more than 10 per cent. Since a former study of the fertilization of the egg of *Bufo* (King, '01) has shown that the egg is normally penetrated by the spermatozoan within three or four minutes after it has been deposited, it is evident that in these experiments the solutions acted chiefly on the zygote and not on the unfertilized egg.

In each case 300 embryos were taken for rearing, and the greater number of these, as shown in table 4, were carried through to metamorphosis and their sex ascertained. Each lot gave a percentage of females higher than that in the control for the series, but well within the limits of normal variation in the percentages of females as shown in table 2. It is doubtful, therefore, if in either case the normal proportion of the sexes was altered by the treatment which the eggs received at the time that they were fertilized.

One lot of eggs, taken from female *b*, was fertilized in a  $\frac{1}{4}$  per cent solution of salt, and another lot was fertilized in a  $\frac{1}{4}$  per cent

TABLE 4  
*Eggs fertilized in hypertonic solutions*

SERIES	SOLUTION USED	TIME OF ACTION	TOTAL NUMBER OF INDIVIDUALS	NUMBER SEX ASCERTAINED	MALES	FEMALES	PERCENT FEMALE	NUMBER MALES TO 100 FEMALES	NUMBER MALES TO 100 FEMALES (CONTROL)
		min.							
A	$\frac{1}{2}$ per cent sugar	30	300	246	112	134	54.47	83.58	110.45
A	$\frac{1}{2}$ per cent salt	30	300	272	130	142	52.20	91.54	
B	$\frac{1}{4}$ per cent sugar	60	400	367	185	182	49.59	101.64	114.10
B	$\frac{1}{4}$ per cent salt	60	450	391	215	176	45.26	121.59	

solution of sugar; in each case the eggs remained in the solution for one hour before being transferred into tap water. These eggs reacted very differently from those that were fertilized in acid solutions (table 3), although they were taken from the same female and fertilized with sperm from the same male. In neither lot was the mortality at the time of fertilization greater than 1 per cent, and only a small number of tadpoles died in the early stages of their development. As shown in table 4, the sex ratio in the individuals that were carried through to metamorphosis was in each instance practically the same as that in the control for the series. These results indicate unmistakably that the solutions in which the eggs were fertilized had no effect on the sex of the tadpoles, although they continued to act on the zygote for nearly an hour.

As indicated in table 4, the results obtained in these experiments offer no evidence that the sex ratio in *Bufo* can be altered by fertilizing the eggs in hypertonic solutions. This negative result may, possibly, be due to the fact that it is not possible to fertilize the eggs in hypertonic solutions that are strong enough to produce any appreciable change in the osmotic pressure.

Keeping eggs out of water for some time after their fertilization was another means employed to cause the zygote to lose water, or at least to prevent its absorption of water, during the early stages of development. This method of experimentation has the very great merit that the eggs are not subjected to the action of any chemical substance that might possibly produce changes in them that would lead to abnormal development and to the early death of the embryos.

The technique employed in the two experiments that were made this year was as follows: On their removal from the uterus of the female the eggs were placed on filter paper and a few drops of water containing spermatozoa were distributed over them with a pipette in as uniform a manner as possible. The excess of water was then quickly drained off, and the eggs were transferred into a moist chamber where they remained for a number of hours before they were allowed to continue their development in water.

With very few exceptions all of the eggs from female *a* that were experimented upon were fertilized, and they began segmenting

fully ten minutes before there was any indication of a division in the eggs of the control lot for the series. When the embryos were removed from the moist chamber and placed in water, seventy-seven hours after the experiment was begun, all of the jelly that had surrounded the eggs had disappeared and the embryos were lying on the nearly dry filter paper from which it took some time to float them off. Out of about 450 embryos that were taken from the moist chamber, 400 were selected for rearing. The tadpoles in this lot were noticeably larger than were any other tadpoles in the series, and they began metamorphosing less than five weeks after the experiment was started. The mortality during the development of the tadpoles was remarkably low, only 4.75 per cent, so that selective mortality could have had very little influence on the proportion of the sexes in the lot of individuals carried through to metamorphosis. In the 381 individuals in which sex was ascertained 275, or 72.33 per cent, were females. This percentage of females is nearly 25 points higher than that in the control for the series (table 1), and much too high to be considered as a chance variation in the normal proportion of the sexes.

In this, as in the other series of investigations made last spring, the experiment was repeated with the eggs from a different female in order to avoid the possibility of drawing conclusions from an unusual sex ratio that might be merely a chance variation. As a check for the experiment made with the eggs from female *a*, a lot of about 400 eggs, taken from female *b*, was fertilized out of water and kept in a moist chamber for fifty hours. In this instance, also, practically all of the eggs were fertilized, and the development of the tadpoles was similar in every respect to that of the tadpoles belonging to the corresponding experiment in series A. The mortality among these tadpoles also was very slight (6.50 per cent), and 374 individuals were carried through to metamorphosis and their sex ascertained. This lot of individuals, as shown in table 5, contained 77.27 per cent of females, which is 30.87 points above that in the control for the series. The sex ratio in this instance, 29.41 males to 100 females, falls far below that in any lot of toads so far examined.

TABLE 5

*Eggs fertilized out of water*

SERIES	TOTAL NUMBER IN-SEX DIVIDUALS	NUMBER ASCER- TAINED	MALES	FEMALES	PER CENT FEMALES	NUMBER MALES TO 100 FEMALES	NUMBER MALES TO 100 FEMALES (CONTROL LOT)
A	400	381	106	275	72.33	38.54	110.45
B	400	374	85	289	77.27	29.41	114.10

In none of the experiments that have been made with the eggs of *Bufo* in order to study the problem of sex-determination have the sex ratios obtained been any where near as low as those indicated in table 5. These results cannot be ascribed to an error in distinguishing the sexes, since the gonads in all of the individuals that were killed three weeks after they had completed their metamorphosis were well differentiated and the sex of the few individuals that died during metamorphosis was shown unmistakably by sections.

It is evident that whatever part selective mortality may have had in producing the unusual sex ratios obtained in various former experiments, it cannot be held responsible for these last results. Had all of the individuals in which sex was not ascertained been males, which of course is very improbable, the resultant sex ratios would still be very much lower than any of those indicated in table 2. The individuals in series A would contain 45.45 males to 100 females; while among the individuals belonging to series B there would be 38.40 males to 100 females: no control lot of individuals so far examined has given a sex ratio of less than 81 males to 100 females.

In these experiments the eggs were not subjected to the action of any chemical substance, but were merely kept out of water for some hours after their fertilization. It is evident, therefore, that the only change that could have been produced in the eggs was a diminution in their water content during the early stages of their development. The eggs probably lost but little water from evaporation during the fertilization period, as they were kept in a moist atmosphere in a closed vessel; but normally, as shown by the investigations of Bialaszewicz ('08), amphibian eggs absorb a considerable amount of water before the appearance of the first

cleavage plane, and such an absorption of water was not possible under the conditions to which these eggs were subjected. Unless by chance, therefore, in picking out the individuals to be reared, I selected in each case tadpoles that would give a great majority of females when developed, I can see no alternative but to assume that sex in *Bufo* can be altered by changing the water content of the eggs at the time of fertilization. The weight of recent experimental and cytological evidence is, however, decidedly against the view that external factors can have any influence whatever in determining sex.

In making these experiments the spermatic fluid was distributed over the eggs within two or three minutes after they had been taken from the female. It is probable, therefore, that each egg was fertilized by the first spermatozoan that reached it, since in such a short space of time the well protected eggs could not lose sufficient water from evaporation to make selective fertilization possible, unless it be that fertilization is normally selective when the eggs of *Bufo* are fertilized. If the male is responsible for sex, each batch of eggs might have been expected to give a nearly equal proportion of the sexes, regardless of the external conditions to which they were subjected at the time of their fertilization; for former experiments have shown that, if the spermatozoa of *Bufo* are dimorphic, both kinds of spermatozoa must be produced in approximately equal numbers in each testicle of every normal male (King, '11). In each case, however, as indicated in table 5, the individuals carried through to metamorphosis contained a proportion of females greatly in excess of that in any control lot as yet examined and much beyond the limits of probable normal variation. The chromosome theory of sex-determination does not, therefore, offer a satisfactory explanation of these results, unless one arbitrarily assumes that the lot of spermatozoa used in fertilizing each lot of eggs happened to contain a much greater number of female-producing spermatozoa than of those that were male-producing.

The results of the experiments in which eggs were fertilized out of water, taken in connection with those obtained when eggs were subjected to the action of hypertonic solutions before fertilization,

strongly suggest that in *Bufo* sex does not depend exclusively on the spermatozoan, but that it is determined by the egg alone, or by both egg and sperm. It would appear, also that sex can be influenced by decreasing the water content of the egg at or before the time of fertilization.

Hertwig's ('06) theory that sex is determined by the mass relation between the chromatin and the cytoplasm seems to offer a tentative explanation of these results, if it is applied to the conditions existing in the zygote and not to those in the ripe, unfertilized egg. Until these experiments have been repeated and extended, however, it will be useless to attempt the formulation of a theory of sex-determination that will explain these results and bring them in harmony with those that have been obtained by other investigators in this field.

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## REINVIGORATION PRODUCED BY CROSS FERTILIZATION IN HYDATINA SENTA<sup>1</sup>

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The full significance of fertilization is far from being clear notwithstanding a vast amount of speculation and observation upon both plants and animals. Darwin observed self-fertilized and cross fertilized plants for several generations and determined that cross fertilization is generally beneficial and self fertilization is injurious. "This is shown by difference in height, weight, constitutional vigor, and fertility of offspring from crosses and self-fertilized flowers, and in the number of seeds produced by the parent plants." He also collected considerable data from breeders showing that the majority of them were of the opinion that cross breeding between individuals of the same race which lived in separated localities, caused an increase of constitutional vigor in the resulting race.

Later Bütschli regarded conjugation in the Protozoa as a process involving rejuvenation and considered fertilization in the Metazoa in the same light. He was followed by Maupas and finally by Calkins who has found that the conjugation of two individuals in a weak race of *Paramoecia* caused a reinvigoration of the race to such an extent that it was able to pass through another cycle of at least 376 generations before it became as weak as the original race from which the two conjugating individuals were taken.

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Although considerable work on the problem of rejuvenescence by fertilization has been done on plants, nevertheless experiments and observations on the multicellular animals in connection with the reinvigoration of the race by fertilization are as yet very few and inconclusive. The purpose of this present paper is to demonstrate that a great amount of rejuvenescence occurs when two weak races are cross bred and that only a small amount of rejuvenescence takes place when each weak race is inbred with itself.

On October 6, 1908, a fertilized egg from a wild culture of the rotifer, *Hydatina senta*, was put into some fresh culture water and on October 12, 1908, a young female hatched from the egg. A pedigreed parthenogenetic culture or race was started from this female and was called race *A*. In the 59th generation of this race *A*, on February 24, 1909, two parthenogenetic sisters were isolated. One became the mother of what has been called the 60th generation of race *A* and the other became the mother of what has been called the 60th generation of race *B*. In other words at the 59th generation the race was split into two sister races. One was still called race *A* and the other was called race *B*. These two sister parthenogenetic races *A* and *B* were kept in syracuse watch glasses. Usually once in forty-eight hours ten daughter-females of each race were isolated, each daughter-female being placed in a separate watch glass. They produced the young females of the succeeding generation. Both races were always fed from the same food culture jars made from a culture of horse manure and water inoculated with bacteria and protozoa. During the first fifteen months, until January, 1910, these food cultures contained a miscellaneous assortment of protozoa but in January, 1910, pure food cultures of the flagellate, *Polytoma*, in horse manure solutions were started and proved so successful that they have been continued to the present time. The special method of making these cultures has been described in a former paper.

The two pedigreed sister parthenogenetic races were continued up to March 3, 1911, at which time race *B* apparently from exhaustion died out in the 384th parthenogenetic generation. However, some fertilized eggs of this race were saved which had been

produced in some minor experiments which had been performed at about this time in February. In this way the race was preserved and used in later experiments in connection with the problem of in- and cross-breeding. The parthenogenetic race *A* is alive at the present time in the 503rd generation, but is in a very exhausted condition.

During the whole period in which the two races were conducted in parallel generations the external factors or environment were as identical as possible. The individuals of each generation were isolated at the same time, put into the same kind of dishes, with approximately the same amount of tap water to which was added the same kind and amount of food from the same food culture jars. They were always kept side by side in the stacked watch glasses, at the same room temperature, and in the same illumination. Some of the time they were kept in a dark room and some of the time in a well lighted room. The greater part of the time they were in Middletown, Connecticut, but during a few weeks of the summer of 1909 they were at Cold Spring Harbor, New York, and also in Vermont. The summer of 1910 they were only out of Middletown two or three weeks when in Vermont. In the summer of 1911 race *A* was in Woods Hole, Massachusetts, and in Vermont for a few weeks.

The criterion selected for deciding whether the races were strong or weak was the rate of parthenogenetic reproduction. This was selected because of simplicity of observation together with its fundamental importance in connection with growth and metabolism.

In order to determine the comparative vigor of the two races *A* and *B* their rates of parthenogenetic reproduction were obtained and compared with the rates of reproduction of two other parthenogenetic races *C* and *D*. Race *C* was started about nine months later than races *A* and *B*, from a parthenogenetic egg of a wild individual which was isolated from a general wild culture of rotifers supposed to have started a few months previously from a fertilized egg. Race *D* was started at the time of the experiments from a fertilized egg of another wild unpedigreed general culture of rotifers which was begun in October of 1908.

In some of the experiments the eggs of the females from the different races were counted at frequent intervals in order to determine whether all the females of the various races produced the same number of eggs in the same period of time. This was not found to be the case for the females of some of the races produced eggs faster than the females of other races but as the eggs of all females of all races hatched in about the same length of time after they were laid, the rates of reproduction were determined by counting the young females in a dish with their mother after a definite period of time had elapsed since the mother was first isolated.

The first series of observations were made during the period in which race *B* was becoming extinct. Many young parthenogenetic females of approximately the same size were isolated from each of the four races at the same time, placed under identical external conditions and their rates of reproduction recorded. Table 1 shows the general results. Race *B* was unmistakably the weakest in that only one female out of sixty isolated was able to live and reproduce, while twenty others lived the normal length of time for individuals of the race, but never laid any eggs. These twenty females developed and produced many eggs in their ovaries but never laid them. The eggs remained inside the body of the female and ultimately seemed to fill the entire animal, crowding and concealing all the internal organs from view. After a time some of these eggs were observed to start development into embryos; but soon the embryos died and many of the egg membranes ruptured and the body of the female became filled with a mass of egg materials from the broken and decomposing eggs. These females finally became larger than normal females: due to this accumulation of unlaid eggs which crowding out the wall of the animal caused the large size. Such females are designated as sterile females. Thirty-nine of the remaining females did not live to maturity probably because of their weak condition. In race *A* forty-six of the young females matured and produced daughter-females at a higher rate than the one female of the *B* race. In race *C* fifty-three of the young females matured and had a higher reproduction rate than either of the races *A*

TABLE 1

Showing the ages and by the reproductive rates, the comparative vigor of four of the pedigreed parthenogenetic races. It also shows that the rate of parthenogenetic reproduction decreases as the race becomes older and if continued for a sufficient length of time the races become extinct.

RACE	RACE DEVELOPED FROM	DATE PARTHENOGENETIC RACE STARTED	TIME OF EXPERIMENT (1911)	AGE OF RACE	PARTHENOGENETIC GENERATIONS	PARTHENOGENETIC ♀s			STERILE ♀s	2-3 PRO-GENY D. ♀s	D. ♀s PRO-GENY D. ♀s	AV. NO. PRO-GENY D. ♀s
						Isolated	Lined	Died				
A.....	Fertil. egg	10-6-'08	2-16 to 3-6	2 yrs. 5 mos.	370-384	60	46	14	0	46	100	4.13
B.....	Fertil. egg	10-6-'08	2-16 to 3-6	2 yrs. 5 mos.	380-384	60	21	39	20	1	2	2
C.....	Parthen. egg	6-16-'09	2-16 to 3-6	1 yr. 8 mos.	262-274	60	33	7	0	53	331	6.62
D.....	Fertil. egg	2-13-'11	2-16 to 3-6	2 to 3 wks.	2-7	60	69	1	0	59	786	13.32

\* Taken from the 56th generation of the A race, February 24, 1909.

and *B*. In race *D* fifty-nine of the young females matured and the reproduction rate was twice that of race *C* and three times that of race *A*.

When the two races *A* and *B* were in the 60th to 80th generations their rates of reproduction were probably very much like that of race *D* although no exact data were taken in this period. However, from general observations made at this time it was clearly seen by the observer that each young female in both of these races under ordinary conditions matured and produced ten or more young daughter-females in forty-eight hours. It was customary in these early generations to take ten young daughter-females from a single mother with which to form the succeeding generation. Later as the generations increased this became impossible and for the isolation of ten young daughter-females at the end of forty-eight hours two mothers were required and later still three mothers were required. At the same time it was noticeable that the females of race *B* in the same length of time were producing fewer daughter-females than the females of race *A*. During the summer of 1910 this was so apparent that difficulty was experienced in being able to isolate ten young daughter-females from both races which were of the same age and size at the end of forty-eight hours. In order to continue these two races in a parallel series of generations by isolations of young females of the same size from both races daughter-females of race *A* were isolated which were produced later in a family, from the tenth to the thirtieth, and the daughter-females from several mothers of race *B* were isolated which were the earliest ones produced in each family. Thus by isolating the daughter-females from near the middle of a family from race *A* and the 'first born' daughter-females from race *B* it was possible to keep the generations of both races parallel.

From table 1 and from these general observations it is readily seen that as the parthenogenetic race became older the rate of reproduction decreased very decidedly and also that the chances for each young female to grow to maturity were lessened. This decrease in the rate of reproduction may not necessarily be due to long continued parthenogenetic reproduction, but rather to

the constant environment of the horse manure food cultures. The influence of the environment upon the race will be considered in a subsequent paper when certain experiments which are in progress now shall have been completed. At present it is useless to discuss this point because of the lack of sufficient data. From the evidence it is also concluded that race *B* has become the weakest or the most exhausted in its general vigor, while race *D* is the strongest and most vigorous.

After the general vigor or vitality of the parthenogenetic races *A* and *B* had been ascertained it was decided to determine whether fertilization within each race would increase its general vitality. Several females from each race were placed in separate new cultures made in small battery jars and allowed to live in them two to three weeks. During this time males appeared and fertilized eggs were produced. After a short time these fertilized eggs from both races *A* and *B* were hatched and a series of parallel observations were made upon the rates of reproduction of the races, from the fertilized eggs, from the original parthenogenetic race, and from the new race *D*. Tables 2 and 3 show the negative influence of inbreeding once in race *A*. Table 4 shows the same result in race *B*.

After these results were obtained it was thought best to ascertain whether or not a second inbreeding of the races which had already been inbred once would reinvigorate them, perhaps by an accumulation of stimuli of some sort which were too weak in the first fertilization to give apparent results. Table 5 gives the data and results of the second successive inbreeding of race *A*. The new race *D* was used as the control or normal race as has been done in the former experiments. Table 6 shows the results obtained from fifty-one females each of which developed from a different fertilized egg of race *B* which had already been inbred once. In neither table is there found any very marked increase of the rate of reproduction. These two races, both of which resulted from a second successive inbreeding of the original races, were continued. Later in the year race *A* produced fertilized eggs which resulted from the third successive inbreeding of the race, and race *B* even was allowed to produce fertilized eggs which

resulted from the fourth successive inbreeding of the race. Previous to this time race *D* had been destroyed and consequently a new race, *E*, was started from a fertilized egg from the same wild general culture of rotifers from which race *D* had been started. This new race was used as the control.

Certain obvious parts of tables 9, 10, 11 and 12 give the data and results of these observations. In these tables it is noticeable that the rates of reproduction of races *A* and *B* have not risen to any marked extent although a slight increase in the rates is apparent. The conclusion may be safely drawn that successive inbreeding of such weak races does not increase their general constitutional vigor to any considerable degree, even though this successive inbreeding is allowed to occur four times, as in race *B*.

As the two sister parthenogenetic races have been demonstrated to be in a weakened state and this weakness has been shown to continue in each race after several successive cross fertilizations have taken place it now remains to show what results are obtained when these two weakened races are allowed to cross breed and reciprocal cross fertilization of the eggs occurs.

The first series of observations on the crossing of the two races *A* and *B* is recorded in table 8. A few females from each of these weakened races were put together into one battery jar which contained a new food culture. Many males soon appeared and after several days eighteen fertilized eggs were taken out and hatched after resting a few days. The rates of reproduction of seventeen of these females were determined and compared with the reproduction rates of ten different females of the new race *D*. The average reproduction rate of these seventeen females was much higher than either of the average reproduction rates of the two parent races *A* and *B* which have been compared with the reproduction rate of race *D* in tables 1 to 4. In fact it even approached closely to the reproduction rate of race *D*. If the records of three of these seventeen females which were probably not the result of a cross fertilization are eliminated the two average reproduction rates are much closer. This great increase in the reproduction rate and its close approximation to that of the control was assumed to be due to a reinvigoration caused by cross fertilization.

TABLE 2  
Table showing by the comparative reproduction rates that inbreeding race A once does not reinvigorate it.

Time 1941	A PARTHENOGENETIC GENERATIONS 406-410 OF A RACE				A 1st FERTILIZED EGG FROM INBREEDING PARTHENOGENETIC GENERATION 370 OF A RACE				D A NEW PARTHENOGENETIC WILD RACE IN ABOUT 40TH-45TH GENERATION, FROM A FERTILIZED EGG			
	♀ ♀s isolated	d. ♀s produced	Av. no. d. ♀s	♀ ♀s isolated	d. ♀s produced	Av. no. d. ♀s	♀ ♀s isolated	d. ♀s produced	Av. no. d. ♀s	Genera- tions		
Exe. 4-30 } A.M. 5-3 } M. 5-3 } M. 5-5 } Exe. 5-5 } Exe. 5-7 } Exe. 5-7 } Exe. 5-9 } Exe. 5-9 } P.M. 5-11 }	9	16	1.77	5	12	2.4	2	18	9	I		
	9	10	1.11	5	14	2.8	5	25	5	II		
	9	16	1.77	5	1	6.2	5	30	6	III		
	10	13	1.3	3	0	0	4	22	5.5	IV		
	10	12	1.2	5	0	0	4	24	6	V		
	Summary	47	67	1.42	23	27	1.17	20	119	5.95		

♀ ♀s.—Females that produce daughter females (d. ♀s).





TABLE 4  
Showing by the comparative reproduction rates that inbreeding race B once does not reinvigorate it.

Time 1911	A PARTHENOGENETIC GENERATIONS 397-401				B 1st FERTILIZED EGG FROM IN- BREEDING PARTHENOGENETIC GENERATIONS 380 OF B RACE				C PARTHENOGENETIC GENERATIONS 207-211 OF C RACE				D NEW PARTHENOGENETIC WILD RACE IN 234 28th GENERATIONS			
	♀ Qs isolated	d. Qs pro- duced	Av. d. Qs	♀ Qs isolated	d. Qs pro- duced	Av. no. d. Qs	♀ Qs isolated	d. Qs pro- duced	Av. no. d. Qs	♀ Qs isolated	d. Qs pro- duced	Av. no. d. Qs	♀ Qs isolated	d. Qs pro- duced	Av. no. d. Qs	GENERA- TION
4-4-6	10	10	1	5	2	0.4	10	70	7.7	5	14	2.8	5	14	2.8	I
4-6-8	10	33	3.3	2	0	0	10	23	2.3	3	28	9.3	3	28	9.3	II
4-8-10	10	10	1	2	0	0	10	10	1	3	47	4.7	3	47	15.7	III
4-11-13	10	6	0	5	0	0	10	9	0.9	3	27	9.0	3	27	9.0	IV
4-14-16	10	25	2.5	5	0	0	5	25	5	3	34	11.3	3	34	11.3	V
Summary	50	78	1.56	19	2	0.10+	45	130	2.88+	25	120	4.8	25	120	4.8	

TABLE 5

Showing by the comparative reproduction rates that inbreeding race A two successive times does not reinvigorate it

A 2nd FERTILIZED EGGS FROM INBREEDING A 1st				D NEW PARTHENOGE- NETIC-WILD RACE BETWEEN 55±-65 GENERATIONS						
Young ♀♀s from different fertil- ized eggs		Parthenogenetic daughter females isolated		Offspring of daughter females		Parthenogenetic				
No.	Time 1911	Time 1911	No.	Time 1911	No.	Av. no. isolated	q ♀s isolated	d. ♀s pro- duced	Av.no. d. ♀s	
1	A.M. 6-20	Eve. 6-24	Large ♀ dead.	No young.						Sterile
2	A.M. 6-22	A.M. 6-24	4	A.M. 6-26	28	7	10	70	7	
3	A.M. 6-22	A.M. 6-24	2	A.M. 6-26	14	7	10	70	7	
4	A.M. 6-22	A.M. 6-27	Large ♀ dead.	No young.						Sterile
5	A.M. 6-23	Eve. 6-28	Large ♀ dead.	No young.						Sterile
6	A.M. 6-23	Eve. 6-28	Large ♀ dead.	No young.						Sterile
7	A.M. 6-23	Eve. 6-28	Large ♀ dead.	No young.						Sterile
8	A.M. 6-23	Eve. 6-28	Large ♀ dead.	No young.						Sterile
9	A.M. 6-23	A.M. 6-25	4	A.M. 6-27	18	4.5	5	55	11	
10	A.M. 6-23	A.M. 6-26	5	A.M. 6-28	28	5.6	5	68	13.6	
11	A.M. 6-24	A.M. 6-26	4	A.M. 6-28	29	7.25	5	68	13.6	
12	A.M. 6-24	Eve. 6-27	3	Eve. 6-29	All dead.					
13	A.M. 6-25	Eve. 6-27	5	Eve. 6-29	30	6	4	52	14	
14	A.M. 6-26	Eve. 6-27	4	Eve. 6-29	20	5	5	60	12	
15	A.M. 6-26	A.M. 6-28	5	A.M. 6-30	37	7.4	5	65	13	
16	A.M. 6-26	Eve. 6-29	Large ♀ dead.	No young.						Sterile
17	Eve. 6-26	A.M. 6-28	4	A.M. 6-30	34	8.5	5	65	13	
18	Eve. 6-26	A.M. 6-29	4	Eve. 6-30	10	2.5	6	39	6.5	
19	A.M. 6-27	A.M. 6-29	3	Eve. 6-30	5	1.66+	6	39	6.5	
20	Eve. 6-27	Eve. 6-30	2	Eve. 7-2	4	2	2	22	11	
21	Eve. 6-27	Eve. 6-29	4	Eve. 6-30	10	2.5	6	39	6.5	
22	Eve. 6-27	Eve. 6-29	3	Eve. 6-30	6	2	6	39	6.5	
23	Eve. 6-28	A.M. 7-2	4	A.M. 7-4	22	5.5	5	63	12.6	
24	Eve. 6-28	A.M. 7-2	Large ♀ dead.	No young.						Sterile
25	Eve. 6-28	A.M. 7-2	4	A.M. 7-4	1	0.25	5	63	12.6	
26	Eve. 6-28	A.M. 7-2	Large ♀ dead.	No young.						Sterile
27	Eve. 6-28	A.M. 7-2	Large ♀ dead.	No young.						Sterile
28	Eve. 6-28	A.M. 7-2	5	A.M. 7-4	30	6	5	63	12.6	
29	Eve. 6-30	A.M. 7-2	5	A.M. 7-4	30	6	5	63	12.6	
30	Eve. 6-30	A.M. 7-2	4	A.M. 7-4	35	8.75	5	63	12.6	
31	Eve. 6-30	A.M. 7-2	3	A.M. 7-4	15	5	5	63	12.6	
32	Eve. 6-30	A.M. 7-2	= ♂♀							
33	Eve. 6-30	A.M. 7-2	3	A.M. 7-4	5	1.66+	5	63	12.6	
34	A.M. 7-2	Eve. 7-3	3	Eve. 7-5	13	4.33+	4	44	11	
35	A.M. 7-2	Eve. 7-3	3	Eve. 7-5	26	8.66+	4	44	11	
36	A.M. 7-2	Eve. 7-3	5	Eve. 7-5	42	8.4	4	44	11	
37	A.M. 7-2	A.M. 7-4	5	A.M. 7-6	44	8.8	4	50	12.5	
38	A.M. 7-2	A.M. 7-4	4	A.M. 7-6	27	6.75	4	50	12.5	
39	A.M. 7-3	P.M. 7-5	3	A.M. 7-7	0	0	5	61	12.2	
40	A.M. 7-3	P.M. 7-5	2	A.M. 7-7	2	1	5	61	12.2	
41	A.M. 7-3	P.M. 7-5	3	A.M. 7-7	5	1.66+	5	61	12.2	
42	A.M. 7-3	P.M. 7-5	2	A.M. 7-7	0	0	5	61	12.2	

# REINVIGORATION PRODUCED BY CROSS FERTILIZATION 349

TABLE 5—Continued

A 2ND FERTILIZED EGGS FROM INBREEDING A 1st				D NEW PARTHENOGENETIC WILD RACE BETWEEN 55±-65 ±GENERATIONS			
Young ♀♀ from different fertil- ized eggs		Parthenogenetic daughter females isolated		Offspring of daughter females		Parthenogenetic	
No.	Time 1911	Time 1911	No.	Time 1911	No.	Av. no. isolated	Av. no. d. ♀s
43	A.M. 7-3	P.M. 7-5	4	A.M. 7-7	15	3.75	5
44	A.M. 7-3	P.M. 7-5	2	A.M. 7-7	0	0	5
45	A.M. 7-3	P.M. 7-5	2	A.M. 7-7	11	5.5	4
46	Eve. 7-3	A.M. 7-5	5	A.M. 7-7	43	8.6	4
47	Eve. 7-3	A.M. 7-5	5	A.M. 7-7	31	6.2	4
48	Eve. 7-3	A.M. 7-5	5	A.M. 7-7	30	6	4
49	Eve. 7-3	A.M. 7-5	5	A.M. 7-7	24	4.8	4
50	Eve. 7-3	A.M. 7-5	5	A.M. 7-7	19	3.8	4
51	A.M. 7-4	Eve. 7-5	4	P.M. 7-7	31	7.75	4
52	A.M. 7-4	Eve. 7-5	4	P.M. 7-7	17	4.25	4
53	A.M. 7-4	Eve. 7-5	4	P.M. 7-7	20	5	4
54	A.M. 7-4	Eve. 7-5	2	P.M. 7-7	9	4.5	4
55	A.M. 7-4	Eve. 7-5	4	P.M. 7-7	8	2	4
56	A.M. 7-4	Eve. 7-5	3	P.M. 7-7	16	5.33+	4
57	A.M. 7-4	Eve. 7-5	4	P.M. 7-7	20	5	4
57			172		864	5.02	217
45			172		864	5.02	68
						2373	10.92
						739	10.86
							Summary
							Summary of the exact no. of fertile in- dividuals used.

TABLE 6

Showing by the comparative reproduction rates that inbreeding race B two successive times does not reinvigorate it

Time 1911	A PARTHENOGENETIC GENERATIONS 413-416			B 2ND FERTILIZED EGGS FROM INBREEDING B 1st			C PARTHENOGENETIC GENERATIONS 303-306 OF THE C RACE			D NEW PARTHENOGENETIC WILD RACE			GENERA- TION
	♀ ♀s iso- lated	d. ♀s pro- duced	Av. no. d. ♀s	♀ ♀s iso- lated	d. ♀s pro- duced	Av. no. d. ♀s	♀ ♀s iso- lated	d. ♀s pro- duced	Av. no. d. ♀s	♀ ♀s iso- lated	d. ♀s pro- duced	Av. no. d. ♀s	
5 14-16	10	11	1.1	2	1	0.5	10	35	3.5	5	12	2.4	I
5 16-18	8	25	3.12	3	1	0.33+	9	58	6.44	5	47	9.4	II
5 18-20	7	7	1	4	8	2	9	87	9.66+	5	53	10.6	III
5 19-22	8	28	3.5	4	13	3.33+	9	72	8	5	64	12.8	IV
Summary....	33	71	2.15	13	23	1.76	37	252	6.81	20	176	8.8	



## 351

TABLE 7—Continued

R 2nd				D					
FERTILIZED EGGS FROM INBREEDING B 1st				NEW PARTHENOGENETIC WILD RACE					
Young ♀s from different fertilized eggs	Parthenogenetic daughter females isolated	Offspring of daughter females		Parthenogenetic					
No.	Time 1911	No.	Time 1911	No.	Av. no.	♀s isolated	d. ♀s produced	Av. no. d. ♀s	
46	Eve. 6-17	A.M. 6-22	Large ♀ dead.	No young.				Sterile	
47	A.M. 6-19	Eve. 6-21	3 Eve. 6-23	0	0	5	66		13.2
48	A.M. 6-19	Eve. 6-21	5 Eve. 6-23	39	7.8	5	66		13.2
49	A.M. 6-19	Eve. 6-21	3 Eve. 6-23	0	0	5	65		13.2
50	A.M. 6-19	Eve. 6-21	4 Eve. 6-23	26	6.5	5	66		13.2
51	A.M. 6-19	Eve. 6-21	4 Eve. 6-23	33	8.25	5	66		13.2
51		127		345	2.71+	159	1622	10.2	Summary
32		127		345	2.71+	54	308	9.4	Summary of the exact number of fertile indi- viduals used.

TABLE 8

*Showing by the comparative reproduction rates that crossing the two races A and B causes a reinvigoration of the ensuing hybrid race*

A 408 X B 2nd FERTILIZED EGGS FROM THE PROBABLE CROSSING OF RACE A. PARTHENOGENETIC GENERATION 408+ AND RACE B, AFTER INBREEDING TWICE, IN A MIXED CULTURE OF THE TWO RACES IN A BATTERY JAR										D NEW PARTHENOGE- NETIC WILD RACE		
Young ♀ ♀s from different fertilized eggs		Parthenogenetic daughter females isolated		Offspring of daughter females			Parthenogenetic					
No.	Time 1911	Time 1911	No.	Time 1911	No.	Av. no.	♀ ♀s isolated	d. ♀s pro- duced	Av. no. d. ♀s			
1	M. 5-22	Eve. 5-24	5	A.M. 5-27	53	10.6	5	62	12.4			
2	M. 5-22	Eve. 5-24	5	A.M. 5-27	62	12.4	5	62	12.4			
3	Eve. 5-22	Eve. 5-24	5	A.M. 5-27	54	10.8	5	62	12.4			
4	Eve. 5-22	Eve. 5-24	3	A.M. 5-27	42	14	5	62	12.4			
5	Eve. 5-22	Eve. 5-24	5	A.M. 5-27	46	9.2	5	62	12.4			
6	Eve. 5-23	Eve. 5-27	5	Eve. 5-29	15	3	5	40	8			
7	Eve. 5-23	Eve. 5-27	5	Eve. 5-29	31	6.2	5	40	8			
8	Eve. 5-23	Eve. 5-27	5	Eve. 5-29	48	9.6	5	40	8			
9	Eve. 5-24	Eve. 5-27	5	Eve. 5-29	31	6.2	5	40	8			
10	Eve. 5-24	Eve. 5-27	5	Eve. 5-29	40	8	5	40	8			
11	Eve. 5-24	Eve. 5-27	3	Eve. 5-29	6	2	5	40	8			
12	Eve. 5-24	Eve. 5-27	3	Eve. 5-29	21	7	5	40	8			
13	Eve. 5-24	Eve. 5-27	4	Eve. 5-29	35	8.75	5	40	8			
14	Eve. 5-24	Eve. 7-28	Large ♀ alive.			No young.				Stenli-		
15	Eve. 5-24	Eve. 5-27	2	Eve. 5-29	15	7.5	5	40	8			
16	Eve. 5-24	Eve. 5-27	4	Eve. 5-29	24	6	5	40	8			
17	Eve. 5-24	Eve. 5-27	3	Eve. 5-29	17	5.66+	5	40	8			
18	Eve. 5-24	Eve. 5-27	5	Eve. 5-29	11	2.2	5	40	8			
18			72		551	7.6+	85	790	9.2+	Summary		
17			72		551	7.6+	10	102	10.2	Summary of exact number of individuals used		
14			59		519	8.79	10	102	10.2	Summary after elimi- nation of nos. 6, 11, and 18.		

TABLE 9  
Showing by the comparative reproduction rates that successive inbreeding of race A three times and of race B four times does not reinvigorate either race, but when the races are cross-bred once reinvigoration of the ensuing hybrid race occurs immediately

Social No.	B 3rd ♀ × A 2nd ♂			A 3rd			B 4th			E		
	THE THIRD SUCCESSIVE INBREEDING OF THE A RACE			THE FOURTH SUCCESSIVE INBREEDING OF THE B RACE			THE FIFTH SUCCESSIVE INBREEDING OF THE A RACE			THE SIXTH SUCCESSIVE INBREEDING OF THE B RACE		
	Time 1911	No.	Parthenogenetic daughter females isolated	Time 1911	No.	Parthenogenetic offspring of daughter females	Time 1911	No.	Parthenogenetic daughter females isolated	Time 1911	No.	Parthenogenetic offspring of daughter females isolated
1	A.M. 9-5	1		A.M. 9-7	1		A.M. 9-7	1		A.M. 9-7	1	
2	M. 9-5	1		M. 9-7	1		M. 9-7	1		M. 9-7	1	
3	A.M. 9-6	1		A.M. 9-8	3		A.M. 9-8	3		A.M. 9-8	3	
4	M. 9-7	1		M. 9-10	10		M. 9-10	10		M. 9-10	10	
5	A.M. 9-8	1		A.M. 9-10	3		A.M. 9-10	3		A.M. 9-10	3	
6	A.M. 9-8	1		A.M. 9-10	6		A.M. 9-10	6		A.M. 9-10	6	
7	A.M. 9-8	1		A.M. 9-10	6		A.M. 9-10	6		A.M. 9-10	6	
8	M. 9-8	1		Eve. 9-10	3		Eve. 9-10	3		Eve. 9-10	3	
9	M. 9-8	16		Eve. 9-10	78		Eve. 9-10	78		Eve. 9-10	78	
10	M. 9-10	6		M. 9-12	42		M. 9-12	42		M. 9-12	42	
		30			153			153			153	
					22			22			22	
					3			3			3	
					25			25			25	
					39			39			39	
					1.56			1.56			1.56	
					29			29			29	
					177			177			177	
					6 10 1			6 10 1			6 10 1	
					Summary			Summary			Summary	



Showing by the comparative reproduction rates that successive inbreeding of race A three times and of race B four times does not reinvigorate either race but when the races are crossed once reinvigoration of the ensuing hybrid race occurs immediately

[illegible]



TABLE 12  
*Summaries of several of the preceding tables showing the slight cumulative effects of successive inbreedings in races A and B*

RACE	PARTHENOGENETIC GENERATION	FERTILIZED EGGS BY INBREEDING	♀ ♀s USED	AV. D. ♀s	
A.....	409-10		55	2.58	Summary of tables 2 and 3 showing effects of one inbreeding in race A
A 1st (370 Par. Gen.)	2	8	57	0.98	
D.....	40-45		35	7.42	
A 2nd.....	1	45	45	5.62	Summary of table 5 showing effects of two inbreedings in race A
D.....	55-65		68	10.56	
A 3rd.....	2	3	53	3.71	Summary of tables 9, 10 and 11 showing effects of three inbreedings in race A
E.....	2-7		63	6.69	
A.....	397-401		50	1.56	Summary of table 4 showing effects of one inbreeding in race B
B 1st (380 Par. Gen.)	2	1	19	0.10+	
C.....	287-91		45	3.88	
D.....	23-28		25	4.4	
A.....	412-416		33	2.15	Summary of tables 6 and 7 showing effects of two inbreedings in race B
B 2nd.....	413-416	33	140	2.23+	
C.....	393-305		37	6.81	
D.....	55+		74	9.1+	
B 4th.....	1-5	3	55	2.36+	Summary of Tables 9, 10 and 11 showing effects of four inbreedings in race B
E.....	3+		63	6.99+	

TABLE 13  
*Summary of several of the preceding tables showing the comparative effects of in- and cross-breeding*

RACE	PARTHENOGENETIC GENERATION	FERTILIZED EGGS	Q'S USED	AV. D. Q'S	
A	413		33	2.15*	Summary of tables 6, 7 and 8 showing the comparative effects of in- and cross-breeding
B 2nd	2	33	140	2.23+*	
A 408 × B 2nd	1	14	14	8.79	
D	40+	1	10	10.2	Summary of table 10 showing the comparative effects of in- and cross-breeding
A 3rd		1	21	4.04	
B 4th		1	21	2.32	
A 2nd ♀ × B 3rd ♂	2	14	54	6.18	Summary of table 9 showing the comparative effects of in- and cross-breeding
E	4+	1	21	6.76	
A 3rd		1	32	3.55	
B 4th		1	34	2.33	Summaries and averages of the above three summarised tables showing by the comparative reproduction rates (Av. d. Q's) the great amount of reinvigoration produced by cross-breeding
A 2nd ♂ × B 3rd ♀	2	10	33	6.21	
E	4	1	42	6.66+	
A	413				
A 3rd	2-10	1	86	3.24--	
B 2nd					
B 4th	2	31	105	2.29	
A 408 × A 3rd					
B 3rd B 3rd	1 and 2	38	101	7.06	
D and E	2.65	2	73	7.69	

\*Taken from another series of experiments made 2-3 days earlier.

Later this assumption was doubted and the possibility was recognized that this reinvigoration might be due to external conditions. Consequently further experiments entailing great care were carried out in which every fertilized egg obtained was known for a certainty to have been fertilized by the sperm of the other race. Separate new cultures of the two races were made in several battery jars at the same time. After a few days many males appeared in both races. Then several thousand females of each race were isolated in separate watch glasses and soon produced both female and male parthenogenetic eggs. These eggs were transferred to other watch glasses containing food culture water and allowed to hatch. At this period of hatching the eggs of both races were continuously watched and the young females and the young males were isolated as soon as they left the egg membrane thus preventing any fertilization by males of the same race. Scores of young females and dozens of young males were isolated from each race and then the young males of one race were placed in the dish containing the young females of the other race. Copulation soon took place and later some of the females at maturity produced the thick-shelled conspicuous fertilized eggs. Such females were readily distinguishable, by the general appearance of the enclosed eggs, from the parthenogenetic females, and were isolated in separate dishes containing food and allowed to produce as many fertilized eggs as possible. By this method several dozens of cross fertilized eggs were obtained. Eggs of race *A* were fertilized by the sperm of race *B* and eggs of race *B* were fertilized by the sperm of race *A*.

Twenty-four of these cross fertilized eggs were hatched thus starting twenty-four separate races. The immediate offspring of each of these races consisting of parthenogenetic females were cared for and the reproduction rates of eighty-four parthenogenetic daughter-females from these were determined. At the same time the rates of reproduction of the *A* and *B* race after the third and fourth successive inbreeding respectively were determined and also that of the control race, *E*. All fertilized eggs in these reciprocal crossbreeding and the third and fourth successive inbreeding experiments of races *A* and *B* were produced in the

same week, rested from two to three weeks, and then were hatched in the same period of time, two to five days. Tables 9, 10 and 11 give the details of the experiments and table 13 shows the general results in a summary of these three tables together with table 8. From these tables it is seen that the reproduction rate of the two crossed races irrespective of the manner of the cross approaches very closely to the reproduction rate of race *E*; while the reproduction rates of each of the two races which had been inbred were very much lower. This seems to be positive proof that in crossing two weak races the resulting race which develops from the cross-fertilized egg has its general vigor or vitality greatly increased.

The high reproduction rates of the two wild parthenogenetic races *D* and *E* and of the parthenogenetic races *A*, *B* and *C* at their beginning, is probably due to the cross-breeding of different races in the same jar. Races *A* and *B* were sister races developing from the same parthenogenetic mother which originally developed from a fertilized egg. If after an interval of about three hundred generations each of these two sister races becomes a distinct race, as is shown by their different rates of reproduction and also by the effects of in- and cross-breeding, it is reasonable to suppose that in a general culture jar standing for two to three years many different races are constantly appearing and inbreeding. Consequently all fertilized eggs taken from such a jar will develop into races each having a high rate of reproduction. It has been previously stated that races *A* and *B* originally developed from one fertilized egg taken from a wild culture jar. Race *C* was taken from a wild culture jar and races *D* and *E* developed from fertilized eggs which were taken from the same general wild rotifer culture jar that was made in October of 1908. All of these races used came from a general culture of rotifers which was originally collected in a certain ditch in Grantwood, New Jersey, in October of 1906.

Shull in his experiments in crossing the New York race with the Baltimore race of *Hydatina senta* caught sight of this same fact that cross breeding two races increases the rate of reproduction but he was not certain of its validity. His table 37 prob-

ably proves it; but as only the descendants of one cross fertilized egg were studied and since there was a lack of all time data and a failure to record the number of parthenogenetic mothers used in each generation, this table can not be taken as very conclusive evidence. This tentative conclusion of Shull's was not known until after nearly all the above results of this paper were ascertained.

Castle and his collaborators in breeding experiments with *Drosophila* state that:

A cross between two races, one inbred for thirty or more generations and of low productiveness, the other inbred for less than ten generations and of high productiveness, produced offspring like the latter in productiveness but not superior to it. The same two races crossed after an additional year of inbreeding (about twenty generations) produced offspring superior to either pure race in productiveness.

This seems to be in the final results a case parallel to that of *Hydantina senta*. When two races have been closely inbred, like brother and sister for many generations or even when two races have been bred parthenogenetically, which is the extreme of inbreeding, for many generations they show in cross-breeding a great increase in productiveness of offspring which is superior to that of either of the parent races.

The effect of inbreeding among animals has been of considerable interest and is of great practical importance; but even at the present time there is much diversity of opinion in regard to the matter. The relation of inbreeding to sterility has been observed in experiments upon mammals by Crampe, Bos, and Guaita; upon birds by Fabre-Donnengue. They all found the relation to be a causal one, continuous inbreeding, as of brothers and sisters, resulting in a decrease of fertility, accompanied more or less by lack of vigor, diminution in size, partial or complete sterility, and pathological malformations.

In inbreeding experiments upon the pomace-fly, *Drosophila*, Castle and his collaborators state that "inbreeding probably reduces very slightly the productiveness of *Drosophila*."

Moenkhaus has recently completed inbreeding experiments upon *Drosophila* in which he has inbred brothers and sisters for

seventy-five generations and found no increase in sterility or a decrease in vigor. Perhaps if these inbreeding experiments can be carried on for two to three hundreds of generations there may appear an increase in sterility and general debility. In *Hydatina senta* at the 75th parthenogenetic generation there was no noticeable decrease of vigor; but much later it gradually appeared as the generations increased and the race became older.

#### SUMMARY

1. Two distinct sister parthenogenetic races of *Hydatina senta* characterized by the difference in their rates of reproduction and general vigor were developed from one original parthenogenetic race under identical external conditions.
2. Races of *Hydatina senta* allowed to reproduce parthenogenetically for 384 generations, extending through a period of twenty-nine months under identical environments, showed a gradual decrease in their rates of reproduction. This was assumed to signify a decrease in the general constitutional vigor or vitality of the race.
3. Successive inbreedings of the weakened parthenogenetic sister races, one to four times, caused a slight increase in their reproduction powers.
4. Reciprocal cross fertilization or cross-breeding of such weakened parthenogenetic sister races of *Hydatina senta* caused a sudden and very pronounced increase in the reproduction rate of the ensuing race. This shows that cross fertilization of the two weakened races greatly reinvigorated both races and probably restored them to their normal vigor which they possessed when they started from the original fertilized egg.
5. The high reproduction rates of new races of *Hydatina senta*, developed from fertilized eggs which were taken from the same general wild culture jar that had been standing at least for twenty-nine months is due probably to cross-breeding of different races. These different races may have been introduced into the culture when it was started or they may have developed since from the original race.



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THE OLFACTORY REACTIONS OF THE PUFFER OR  
SWELLFISH, SPHEROIDES MACULATUS (BLOCH  
AND SCHNEIDER)

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The opinion has long been held that the olfactory organs of fishes are concerned with a sense of smell, and that many species make considerable use of this sense in locating food. Only recently, however, has this view been substantiated by physiological evidence. Parker ('10) was the first to describe reactions of fishes which were unquestionably dependent on the stimulation of the olfactory apparatus by odorous substances. The species studied was the fresh water catfish, *Amiurus nebulosus*. The following year Parker ('11) tested the common killifish, *Fundulus heteroclitus*, and Sheldon ('11) the smooth dogfish, *Mustelus canis*, for the sense of smell, and obtained marked response to olfactory stimuli from each. A few weeks spent at the Biological Laboratory of the United States Bureau of Fisheries at Woods Hole, Massachusetts, afforded me opportunity to study the sense of smell in the puffer, *Spheroïdes maculatus*, with the following results. I wish to express my thanks to Dr. F. B. Sumner, Director of the laboratory, for many kindnesses received during my stay.

The olfactory apparatus of the puffer is not of the type most commonly seen in the higher fishes. Each nasal chamber occupies the interior of a papilla which rises about 4 mm. above the upper surface of the snout, antero-medial to the eye, and is provided with two small circular apertures, one anterior in position, and the other situated at the end of a rather poorly marked cylindrical extension directed laterad. Both apertures seem to be constantly open.

By testing a resting fish with carmine suspended in water, I was unable to discover any evidence of a current passing through the olfactory chambers, neither of the intermittent type dependent on respiratory movements common in many fishes, nor of the continuous kind produced by cilia, as described by Parker ('10) in the catfish. If, however, a colored solution is gently forced into one of the olfactory apertures by means of a pipette, it readily passes through the chamber and out the other aperture. I am led to conclude, therefore, that the forward locomotion of the puffer forces water through the anterior openings of the nasal chambers and out the lateral ones, and at that time conditions are most favorable to the stimulation of the olfactory cells by odorous substances. That, in truth, the puffer is seldom at rest, when in captivity at least, is readily apparent after a few hours observation of its habits in a large aquarium. The elevated position of the nasal chambers is well adapted to the formation of water currents through them by forward locomotion. When a fish is swimming rapidly back and forth in an aquarium the olfactory organs become directed backward slightly, as it progresses through the water, but become erect as it turns in its course. At first, I believed that this inclination of the nasal organs was indicative of the force of impact of the water against them. I subsequently discovered, however, that this is not necessarily so, as the same result could be obtained by making a threatening gesture in front of the fish.

Preliminary tests to determine whether the puffer would react to concealed food were begun upon eight to twelve fishes, which occupied one of the large observation aquaria of the Station. The method of experimentation was essentially like that of Parker ('10, '11), and Sheldon ('11). Two cheese cloth packets of similar appearance, one containing meat of the smooth dogfish, *Mustelus canis*, and the other filled with cheese cloth, were suspended some distance apart in the aquarium. The presence of food in one of the packets could be detected by the fish only through the stimulation of its chemical sense organs by material emanating from the meat. In several tests the packet containing meat was quickly seized and bitten open, whereas the other, although some-

times bitten, received less attention and never was opened. When packets made of cotton cloth were substituted for those of cheese cloth, similar results were obtained; i.e., the one with meat was bitten open, the powerful jaws of *Spheroides* cutting through the cloth as if it had been tissue paper. From these tests it became evident that, in order to obtain any extended series of reactions to the packets, they must be constructed to withstand a severe biting as well as permit the escape of odorous material. This end was accomplished by covering with cheese cloth a pair of tea strainers made of tin and fine wire netting, one of which was filled with dogfish meat, and the other with cheese cloth. The fish were not adverse to biting such an object, which was at the same time flexible and impossible to tear apart, and, accordingly, they were used throughout the experiments to be described.

With two packets reconstructed in this manner I again proceeded to test the fish, about a dozen in number. For fifteen minutes after the packets were suspended in the aquarium, the puffers bit actively at both, but decidedly more at the one containing meat. At the end of that time, they were observed for one hour, and a record was kept of the number of times each packet was bitten, the relative positions of the two being changed every fifteen minutes. The packet containing meat received 42 bites, the one with cheese cloth 4. These tests show conclusively that the puffer is able to discover concealed food.

That sight plays an important part in the search for edible substances is made clear from the fact that the packet of cheese cloth is occasionally seized by the hungry fish. Moreover, if a wad of filter paper attached to the end of a wire is drawn through the water, it is pursued and taken into the mouth as eagerly as if it were meat. But, whereas the meat is always swallowed, the filter paper, although often drawn into the mouth several times, is ultimately discarded. Similar reactions were observed by Parker ('11) in the killifish. That sight will not explain their final discrimination between edible and inedible material is quite evident.

I next planned a series of experiments to ascertain the part played by the olfactory apparatus in the reactions of the fish to

hidden food. Four puffers were isolated in an aquarium, and their normal reactions to the two packets were first recorded. As in previous experiments, fresh dogfish meat was always used for food, and the positions of the baited and unbaited packets were exchanged every fifteen minutes. During the first test hour, when the fish were very hungry, the packet with meat was bitten 119 times, and the cheese cloth packet 18 times. At the end of the experiment the fish were fed, and three days later they were again tested for an hour: 67 bites at the baited packet, and 8 at the other one resulted. It now became necessary to eliminate the olfactory organs, to repeat the tests with the packets, and compare the results with those set forth above. To render functionless the olfactory apparatus of *Spheroides* was a comparatively easy task, involving no cutting of nerves or stitching together of nares. A silk thread, tied by a single knot around each organ, contracted the olfactory chambers so as to prevent effectually any flow of water through them.

About two hours after the close of the test last described, the nasal organs of the four puffers were tied in this manner. An hour later they were snapping small pieces of dogfish meat from the end of a wire in perfectly normal fashion, and soon afterward I tested them for an hour with the two packets. At no time did they pay any attention to either, although they eagerly seized small pieces of meat dropped into the aquarium, or offered to them on the end of a wire. Eighteen hours later the test was repeated with similar results. As the packets were being suspended in the aquarium, the one containing cheese cloth was bitten twice by one of the fish, an evident visual reaction, but at no other time during the hour was either touched. The fish swam about in a characteristic manner, and, on being tested with meat fragments, showed they were hungry. There was nothing in their behavior to indicate that the contracted state of their olfactory organs was in itself at all disturbing. At the conclusion of the test the threads were removed, and, as might be expected, the nasal organs appeared considerably distorted. On the following day the fish failed to react to the packets. Two days later, however, after sufficient time had elapsed for the recovery of the injured parts,

reactions to the packets again resulted, characterized by normal discrimination between the two. In a fifteen minute test the baited packet was bitten 28 times, and the other one twice.

These experiments show that reactions to concealed food cease when the olfactory apparatus is rendered inoperative, and are resumed only when the organs again become functional.

A week later the foregoing experiments were repeated, and an attempt made to hasten the recovery of the olfactory organs by shortening the time during which they were to be tied. Two of the four puffers previously tested were isolated in an aquarium and allowed to become hungry. They then reacted in an essentially normal way to the two packets, biting the one with meat 34 times, and the other 10 times in one hour. For eleven minutes after the packets had been suspended in the aquarium, no apparent attention was given them, although both fish were passing and repassing them constantly. Suddenly one of the puffers approached the packet containing meat and bit it. It was a reaction which any unprejudiced observer would have called olfactory. After this test, the nasal organs were tied, and about two hours later the fish were again tested for an hour. The thread which surrounded one of the organs dropped off shortly before the test was made. Neither of the packets was touched, although before and after the test both fish ate pieces of meat from the end of a wire in their habitual way. The threads were immediately removed, and, on the following day, they were given two one-hour tests with the packets. No reactions to either resulted. Forty hours after untying the threads they were again tested. One of the fish bit 8 times at the packet with meat and 5 times at the one made of cheese cloth, whereas the other fish ignored both. Each ate actively from the end of a wire at the conclusion of the test.

Subsequent examination of the condition of the nasal organs of these two puffers showed that such behavior might have been expected, if concealed food is scented by means of these organs. Both organs of the fish which failed to react were so badly crushed by the second tying that they could not have been functional afterward. The second puffer, however, showed one organ substan-

tially normal, the other somewhat distorted, a condition of the olfactory apparatus which might well allow stimulation of sufficient strength to call forth a response as described above; one weak in character, in which discrimination between the two packets was less marked than normally.

The tests here recorded show that the puffer approaches and bites a packet containing concealed food many more times than it does one filled with cheese cloth; secondly, that these reactions cease when the olfactory apparatus is rendered inoperative (although pieces of meat are eagerly taken when seen) and, thirdly, that the ability to discriminate between the two packets is regained when the olfactory apparatus again becomes functional.

I, therefore, conclude that *Spheroides maculatus* responds to a stimulation of its olfactory apparatus by substances in dilute solution emanating from concealed dogfish meat, and, that it discovers hidden food by the sense of smell.

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## SIZE INHERITANCE IN DUCKS

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The inheritance of size in animals is a question of great theoretical interest, but difficult to analyze.

Lock ('06) in the case of maize showed that height of the plant is not inherited as a simple Mendelian character. Castle ('09) showed the same to be true of the weight and of various skeletal dimensions of rabbits, and characterized such inheritance as blending.

Ghigi ('09) referring to a cross which he made between Paduan fowls and bantams stated that in size of body and of eggs produced the ( $F_1$ ) cross-bred individuals were intermediate between the parent races, and that later generations showed no tendency to return to the conditions found in the parent races. The number of animals studied by Ghigi was small and no great stress was laid upon the point of non-segregation.

Emerson ('10), however, after a more detailed and exact study of the inheritance of height in maize, and of several other size and shape characters in gourds, found that while  $F_2$  was strictly intermediate between the parents and no more variable,  $F_2$  showed a greatly increased variability, which he interpreted as "merely a mathematical way of expressing the fact that the  $F_2$  individuals exhibit marked segregation of size and shape characters." Such an interpretation was made possible by the discovery by Nilsson-Ehle ('09) and by East ('10) that a Mendelizing character may have multiple germinal representation, in which case, though physiologically a single unit-character, it may produce dihybrid, trihybrid, or even more complex Mendelian inheritance ratios

<sup>1</sup> Contribution No. 12.



in  $F_2$ . If in such cases *dominance* is lacking, an apparently blending inheritance results, attended in  $F_1$  by *no increase of variability*, but in  $F_2$  by *greatly increased variability*.

Now this, as Emerson has shown, is exactly what happens in size inheritance in plants. Such cases are therefore open to interpretation as Mendelian inheritance without dominance, in which more than a single unit character is involved. The theoretical aspects of the matter have been fully discussed by Lang ('10) and Castle ('11).

The following preliminary results (summarized in table 1) from a cross between two different size races of ducks show indications of segregation in body weight. As it will be some time before further data can be added, it is thought worth while to record the experiment as far as it has progressed.

An experiment of this sort, based, as it must be, on the perfect health of the animals, is necessarily subject to a serious source of error. Larger numbers than have yet been obtained are therefore desirable, and it is hoped that these may be had in future seasons. The season of 1911 was very dry and hot and may have had some effect on the variability of the  $F_2$  generation. It was certainly not favorable toward fertility in duck eggs. The  $F_1$  generation is much too small and an attempt will be made to obtain larger numbers, using as mothers random females from the small race. Tarsus and bill measurements will also be taken to supplement the weights of the animals.

#### MATERIAL

Ducks were chosen for this purpose because it is possible among them to obtain two races greatly different in size, and yet producing fertile hybrids. Important also is the fact that the young can be raised in large numbers during a single definite growth season. Maturity is reached quickly, and entire lots can be killed and examined at the same age. It is possible also to raise different groups or generations during the same season, thus providing a check upon environmental effects.

The large French Rouen duck was chosen for the large parent, and the common domestic mallard for the small parent.

TABLE 1  
*Synopsis of experiment*

	GROUP	AGE	NUMBER OF MALES OF FEMALES	MEAN WEIGHT MALES	MEAN WEIGHT FEMALES	STANDARD DEVIATION		C. V.	
						MALES	FEMALES	MALES	FEMALES
				gms.	gms.				
Large race (Rouen)	Original stock...	1½ years	2	2790	2537				
	Second generation...	5 months	4						
	Third generation...	5 months	2	2535	2306				
	Second and third generation combined	5 months	6	2250	2149				
Small race (Mallard)	Original stock...	5 months	8	2321	2244	189	193	8.15	8.63
	Second generation...	5 months	About 30	1068	928	119	76	11.14	8.18
	Entire F <sub>1</sub> generation...	5 months	43						
	Entire F <sub>2</sub> generation...	5 months	10	1065	1587	97	54	5.82	3.43
F <sub>1</sub>									
F <sub>2</sub>									
		134 to 151 days	17	1781	1634	224	181	12.57	11.07

The original Rouen stock consisted of two males and four females. They were obtained from a dealer in April, 1910, but were not weighed until the following fall. During the summer of 1910 two males and five females were raised from the original stock, while in 1911 six males and nine females were brought to maturity from the same source. These twenty-two birds (second and third generation, Rouen stock, table 1) were killed and weighed at the same time as the  $F_1$ 's and  $F_2$ 's, so that a definite control might be obtained. The mean weight of the Rouen race was calculated from these twenty-two individuals. For the males, it was found to be 2321 grams; for the females, 2237 grams. The original Rouens were not included in this calculation, because when weighed for the first time, at one and one-half years of age, they were very fat and not directly comparable with their offspring. Their weight was 16 per cent greater than the

TABLE 2  
*Weights of pure large race, Rouen*

		MALES		FEMALES
		Age	Grams	Grams
Original stock .....	1½ years		2600	2220
			2980	2290
				2770
				2870
			2470	2220
Second generation (1910) .....	5 months		2600	2300
				2370
				2420
				2670
			1980	1885
Third generation (1911) .....	5 months		2162	1990
			2252	2095
			2260	2165
			2335	2175
			2512	2200
				2202
				2230
				2460

mean of all their offspring. It seems probable that the mean weight of the Rouen stock as here used is rather too small. Maturity is probably not reached quite as soon by this large breed as by the smaller breeds and the very vigorous  $F_1$  cross-bred birds.

The mallard stock was obtained from one hundred and twenty-five eggs received from a game preserve in New Jersey in the spring of 1909. From these eggs sixty-five birds were raised to maturity. In 1910 part of this original stock produced one hundred and sixty mallards which were raised to maturity. Most of these were killed off and weighed in three lots, October 13, October 22 and November 10 (table 3). A comparison of the weights showed that

TABLE 3  
*Weight of pure small race, mallard*

AGE	MALES	FEMALES
	Grams	Grams
	845	819
	854	864
	892	874
	896	855
	910	895
	916	896
	934	970
	944	994
	973	1005
	1024	1039
4½ months.....	1064	1048
	1074	
	1094	
	1096	
	1100	
	1112	
	1115	
	1140	
	1174	
	1180	
	1222	
	1313	
Mean.....	1039	985

TABLE 3—Continued

	MALES		FEMALES	
	Grams		Grams	
4½ months.....		870		770
		900		855
		905		855
		920		840
		948		860
		950		875
		990		890
		990		942
		1080		945
		1100		950
		1105		952
		1105		960
		1110		963
		1115		970
		1115		970
		1130		985
		1145		993
		1150		1002
		1155		1020
		1180		1040
		1185		1045
		1185		1090
		1190		1090
		1225		
		1250		
Mean.....		1076		909
5½ months.....		895		920
		935		925
		960		925
		1030		956
		1030		960
		1035		960
		1130		980
		1160		1035
		1160		1045
		1180		
		1250		
		1312		
Mean.....		1089		967
Mean all ages.....		1068		928

the birds had practically gained their adult size by October 22. The mean weights of the three lots is as follows.

SEX	OCTOBER 13	OCTOBER 22	NOVEMBER 10
Males.....	1039	1076	1089
Females.....	935	937	967

This stock of mallards is the pure semi-domestic *Anas boschas* used in the English game preserves. They are perhaps slightly larger than pure wild mallards, which average about 1000 grams for the males, depending somewhat on food supply; (no large series of the weights of wild mallards is at hand).

#### MATINGS

In the spring of 1910 three female mallards taken at random from the original stock were mated with one of the Rouen stock males. At the same time two female Rouens were mated with a male mallard, but the two settings of eggs which were obtained from this last mating proved to be entirely sterile, due undoubtedly to the small size of the male bird. While on the subject of this attempted reciprocal cross, it might be well to state that there is probably little or no error introduced into the experiment on account of a difference in size of the eggs in the two races. The lengths of seven Rouen and seventeen mallard eggs were measured in 1909. There is a good deal of variation in the eggs of both strains, and an overlapping of the two variation curves. The Rouen eggs are roughly 2.40 inches in length and the mallard eggs 2.25 inches in length.

The  $F_1$  eggs from the mating male Rouen  $\times$  female mallard were set in two lots and hatched on the same date, May 29. They were reared under hens in exactly the same place and under the same conditions as the two parent classes. Both this group and the  $F_2$  birds were banded. These  $F_1$  birds, ten males and three females, were weighed October 29, at the age of five months. The class is obviously too small, but as far as it goes it shows a remarkable uniformity. Between the largest and smallest males

there is a difference of only 200 grams. The coefficient of variability (c. v) is 5.82 for the males and 3.43 for the females.

In 1911 an attempt was made to raise another lot of  $F_1$ 's but only two fertile eggs were obtained and these were discarded.

The  $F_2$  generation (table 4) was obtained by mating the three  $F_1$  females, Nos. 83, 101 and 106, to male No. 105. This male is slightly above the mean size of the  $F_1$  males, which fact perhaps accounts, in part at least, for the larger size of the  $F_2$  mean, as compared with that of the  $F_1$ . The eggs from this mating were set in three lots, May 29, June 8 and June 15. The entire class was killed and weighed on October 29. The ages therefore varied from one hundred and thirty-four to one hundred and fifty-one days. The mean weight of the seven youngest males, hatched June 15, is 1738 grams, as against 1863 grams for nine males hatched May 29, a difference of about 7 per cent. This shows that adult weight is very nearly reached at four and one-half months of age.

A glance at table 4 shows the very remarkable variation which these  $F_2$ 's exhibit. There is an actual range of variation in weight of 887 grams among the males and of 650 grams among the females. The smallest male, No. 158, is of substantially the same weight as the largest pure mallard. The largest  $F_2$  male, No. 175, is only slightly smaller than the mean of the pure Rouen males for 1911, the difference being only 2 per cent.

The  $F_2$  coefficient of variation is 12.07 for the males as against 5.82 for the  $F_1$  males, while the  $F_2$  females have a coefficient of variation of 11.07 as against 3.43 for the  $F_1$  females.

It is interesting to note that the coefficient of variation is larger in the males than in the females, in all groups except the pure large race, where the reverse is the case.

#### GENERAL CONSIDERATIONS

A fuller statistical analysis of the foregoing data may help to make their significance plain. For this purpose the observed weights may be classified in a variation table. Since males are on the average heavier than females, it is evident that separate tables must be made for males and females. Let us take the mean

TABLE 4  
*Weights of F<sub>1</sub> Animals when five months old, 1910*

MALES		FEMALES	
No.	Grams	No.	Grams
84	1470	83	1570
85	1660	101	1530
86	1600	106	1660
87	1670		
100	1740		
102	1740		
103	1650		
104	1770		
105	1720		
107	1630		
Mean.....	1665		1587

TABLE 5  
*Weights of F<sub>2</sub> Animals, 1911.*

MALES			FEMALES		
No.	Age, days	Grams	No.	Age, days	Grams
158	151	1320	161	151	1325
164	151	1967	165	151	1527
166	151	2060	167	151	1875
170	151	1885	168	151	1697
171	151	2035	169	151	1975
175	151	2207	172	151	1295
176	151	1815	173	151	1603
177	151	1847	180	151	1895
178	151	1632	181	151	1650
189	151	1725	182	151	1675
200	141	1482	183	151	1645
214	141	2007	186	151	1717
215	141	1583	187	151	1672
217	134	1760	199	141	1410
223	134	1667	222	134	1572
224	134	1660	229	134	1615
303	134	1720			
Mean.....		1781			1634



weights of each pure race as class means in a variation table, which shall contain a convenient number of intermediate classes, say seven. Tables may thus be constructed separately for each sex (tables 6 and 7).

In both tables the variation of the pure races is seen to be about classes 2 and 10 as their respective modes. Indeed the tables were constructed with that end in view, the observed means of the weights being made the means for classes 2 and 10. Since further the number of intervening classes is the same in both tables, these tables may legitimately be combined, class by class, to obtain a table of weight distribution for both sexes, which however will be free from any serious error<sup>2</sup> due to the fact that the two sexes differ in size. Such a combination table is table 8.

From an examination of these tables it is clearly seen that (1) the  $F_1$  animals vary closely about the middle class, 6, exactly intermediate between the parent races. The extent of variation of the  $F_1$  animals is small. (2) the  $F_2$  animals vary about the same intermediate class, 6. The amplitude of variation of the  $F_2$  animals is greater than that of the  $F_1$  animals, but does not extend beyond the nearer limit of the respective grand-parental races. In the 33  $F_2$  animals studied no variate occurs which is as small as the mean of the small race or as large as the mean of the large race. No case of *complete segregation* occurs. (3) The increased variability of  $F_2$  as compared with  $F_1$  may be regarded as due to partial segregation of genes having multiple representation in the gamete, or as due to modification of gametes in other ways as a result of their association in  $F_1$  zygotes. The evidence at present available is insufficient to decide between these contrasted views.

In conclusion, I wish to express special thanks to Professor Castle for valuable help in correlating the results of this experiment.

<sup>2</sup> This difficulty was met by Galton ('89, *Natural inheritance*) in a different way, which however would for our present purpose, it is thought, be less accurate as well as more laborious. Finding that the average male measurement was greater than the corresponding average female measurement he ascertained the ratio of the two to each other and then converted all female measurements into equivalent male measurements by multiplying by this ratio.

TABLE 6.  
*Weight in grams of male ducks*

CLASS LIMITS	835- 930		991- 1146		1047- 1312		1393- 1538		1439- 1614		1635- 1770		1771- 1926		1927- 2082		2083- 2238		2239- 2394		2395- 2550		2551- 2706		2707- 2862	
	1	2	3	4	5	6	7	8	9	10	11	12	13													
Class no. ....	1	2	3	4	5	6	7	8	9	10	11	12	13													
Rouen .....																										
Mallard .....	20	23	14	2									1													
F <sub>1</sub> .....					2	8																				
F <sub>2</sub> .....				1	2	6	3	5																		

TABLE 7.  
*Weight in grams of female ducks*

CLASS LIMITS	683- 846		847- 1010		1011- 1174		1175- 1338		1339- 1502		1503- 1666		1667- 1830		1831- 1994		1995- 2158		2159- 2322		2323- 2486		2487- 2650		2651- 2814	
	1	2	3	4	5	6	7	8	9	10	11	12	13													
Class no. ....	1	2	3	4	5	6	7	8	9	10	11	12	13													
Rouen .....																										
Mallard .....	3	31	9			3							1													
F <sub>1</sub> .....																										
F <sub>2</sub> .....				2	1	6	4	3																		

TABLE 8  
*Weight distribution of male and female ducks combined*

Class number.....	1	2	3	4	5	6	7	8	9	10	11	12
Rouen.....								3	3	9	5	1
Mallard.....	23	51	23	2								
F <sub>1</sub> .....					2	11						
F <sub>2</sub> .....				3	3	12	7	8				

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# CAN THE SPERMATOZOÖN DEVELOP OUTSIDE THE EGG?

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ELEVEN FIGURES

## I. INTRODUCTION

The experiments on artificial parthenogenesis have shown that an egg which *naturally* cannot develop without sperm can be caused artificially to develop without being fertilized by sperm. It is natural to raise the question whether a spermatozoön can be caused to develop into a larva without an egg. The experiments on merogony prove that a fragment of the egg deprived of a nucleus can develop into a larva if a spermatozoön enters the egg; this shows that the egg nucleus is not necessary for the development. The phenomena of merogony do not yet prove that the spermatozoön alone can give rise to an embryo. There are several reasons for doubting this possibility. In the first place we have reason to assume that the protoplasm of the egg is the embryo itself. If this be correct we can understand that the spermatozoön might be able to transmit a number of characters to the offspring, without possessing the possibility of becoming an embryo or creating one outside of an egg.

In the second place it is possible that only the protoplasm contains the apparatus necessary for nuclear and cellular division and that a spermatozoön is not able to create this apparatus. In the third place it is possible that the egg protoplasm of each species contains nutritive material and enzymes of so specific a character that it is not likely that we are able to imitate it in the near future. The attempt at causing the spermatozoön to develop without an

egg is at the same time an attempt to test the validity of the reasons which seem to speak against this possibility.

There is only one paper dealing with this problem, by J. de Meyer.<sup>1</sup> He raised the question whether it is necessary that the spermatozoön should come in contact with the cytoplasm of the egg in order to undergo the first phases of its normal evolution. He used the sperm of *Echinus microtuberculatus*, which he placed in sea-water containing an extract of the eggs of the same species and found that under these conditions the spermatozoa swelled so as to lose completely their normal appearance. The tail remained unchanged, but the cytoplasmic covering of the head, the middle piece, and the chromatic portion of the head all seemed to swell; and in some cases an indistinct vesicular structure was seen which stained a little stronger than its surroundings, and seemed to be a nucleus. He concludes that incomplete as his results may be, they give a right to conclude, "that the male just as the female cell is capable of evolution under the influence of external agencies" (p. 94).

## II. MATERIAL AND METHODS

Our own experiments were carried on on the sperm of the fowl. The sperm was removed aseptically. Only the sperm contained in the lower portion of the vas deferens was used. It was kept in a sterilized moist chamber at about 39° C., but was always used soon after its removal from the animal, not later than three hours after it was taken out. The media used for the culture of the spermatozoön were: egg yolk, egg albumen, chicken blood serum and  $\frac{M}{6}$  and  $\frac{M}{16}$  Ringer solutions. Slides, cover glasses and instruments were sterilized in a flame and small hanging drops of the various media were inoculated with the spermatozoa. The cover glasses were inverted over hollow slides and sealed with a vaseline and paraffine mixture. In a few cases the eggs were broken into glass vessels and small quantities of sperm injected into the yolk with a capillary pipette. After stated intervals yolk and sperm were taken out for examination with a capillary pipette.

<sup>1</sup> J. de Meyer, *Arch. de Biologie*, vol. 26, p. 65, 1911.

## III. OBSERVATIONS ON LIVING MATERIAL.

When the spermatozoa of the fowl are observed in a hanging drop of white of egg, kept at about 40° C., the first change is seen after fifty or sixty minutes. It consists in the collection of a small amount of some substance having a low refractive index about the middle pieces of some of the spermatozoa. In favorable cases as many as 60 per cent of the spermatozoa may undergo this change. At this time many of these spermatozoa are still swimming. During the course of the next few hours these lowly refractive areas increase in size until they are about half as long as the sperm head and acquire a fairly distinct ellipsoidal outline. Then in many cases the sperm head can be seen to be bent in a horse shoe or spiral shape, and to be included in the wall of the vesicle, which has now become spherical, while the tail of the spermatozoon still remains unchanged or has disappeared without taking any part in the transformation. The next change is an increasing indistinctness in the sperm head, and an increasing refractive power of the whole vesicle so that it can hardly be discriminated at all in the albumen. It is not possible to follow the process farther in unstained material.

In some cases these vesicles instead of being spherical stretch out along the whole side of the sperm head, or may become entirely disconnected from the spermatozoon.

If yolk is used as a culture medium for the sperm essentially the same phenomena occur; and in the various Ringer solutions vesicles containing the sperm heads are also formed, but in the Ringer solution, as a rule, the steps in the formation of these vesicles could not be seen without staining.

## IV. OBSERVATIONS ON PRESERVED MATERIAL

When the hanging drops are fixed in Flemming's fluid and stained and examined in Herla's vesuvin and malachite-green mixture, it can be seen that in its early stages the vesicle has distinct walls and a homogeneous unstained fluid of a low refractive index in its interior. This fluid is possibly water and this would

account for the fact that the vesicle is conspicuous in albumen and yolk, but invisible in Ringer solution.

The vesicle seems to be formed by the imbibition of water by the very thin protoplasmic envelope of the sperm head and middle piece. For after the formation of the vesicle, head, tail and middle piece are, so far as can be seen, unchanged (fig. 1). In many cases the vesicle is seen at the front end of the spermatozoon. Such cases result from the bending of the spermatozoon in the middle piece region, as the series illustrated in figs. 1, 2 and 3 show. The maximum development of the unchanged vesicle is shown in fig. 4.

When the vesicle has reached its full size the material of which its surface is composed seems to wet the sperm head very easily. For in the next stage the sperm head is in contact with the wall of the vesicle along its whole length, and the vesicle has usually assumed a more or less spherical shape (figs. 5 to 8).

Up to this point the transformations were found to take place in the same way in all the media employed, but in the various Ringer solutions the transformation went no farther than this even when the spermatozoa were left in the solutions for forty-eight hours and longer.

In the yolk and albumen, however, the development toward the formation of a nucleus went a little farther. In these media solution of the sperm head took place, which seemed to begin as soon as the head was drawn into the vesicle. For in these media stages like figs. 7, *a*, *c*, *d*, *e*, were quite difficult to find, while in the Ringer solutions such stages, and those in which the sperm head within the vesicle was entirely unchanged were the most frequent transformations observed (figs. 5 and 6).

In preparations that were fixed after the spermatozoa had been in contact with the yolk or albumen only two or three hours the most frequent transformation of the vesicles observed is one in which the head has entirely disappeared while the whole vesicle takes a rather dilute nuclear stain. In a few cases nuclei of this type still show remnants of the sperm head as in fig. 7, *b* and *c*. These appearances would seem to indicate a solution of the chromatin of the sperm head in the contents of the vesicle.

After the spermatozoa have been left in contact with the culture medium for about eighteen hours no more, or but very few, of these uniformly stained vesicles are to be found. But there are many fairly normal looking nuclei in which the chromatin is all present in the shape of discrete particles resting on the nuclear wall, and in which no linin, or but very small amounts of it, can be seen (figs. 8 to 11). The principal reason for believing that a certain amount of linin is present, even if it is obscured by the chromatin, is that some vesicles are seen, which seem to have broken away from the spermatozoa before they came in contact with the sperm head, and which do not contain any chromatin at all. In these a few strands of linin-like substance may usually be seen traversing the interior of the vesicle; and it is likely that these strands are also present in the other vesicles but cannot be made out on account of the chromatin.

It would seem probable that the chromatin in these nuclei is derived by a condensation of the uniformly distributed chromatin of the previous stage, though it is possible that in a certain number of cases the sperm head breaks up into chromatin particles without a previous complete solution.

Ordinarily no signs of either protoplasm or sperm tails are to be seen in connection with these nuclei but occasionally both may be observed, as in figs. 8*b* and 10*a*; and in several cases it was seen that the middle piece had not been incorporated within the nucleus or vesicle but could be distinctly made out in the tail attached to the vesicle.

When the preparations are fixed in Flemming's fluid, stained with Czaplewsky's carboic gentian violet, dehydrated and mounted in balsam, clearer pictures of the completed nuclei were obtained (figs. 9 to 11) but the series of intermediate stages in the formation of these nuclei seemed to be entirely different. It was possible to make out a connected series of transformations of the sperm head into nuclei, but since this series contained none of the vesicles so characteristic for the living and glycerine material it must be concluded that this series is composed mainly of artefacts resulting from the shrinkage of the vesicles.



In these balsam preparations the first change which seemed to take place was a broadening of the sperm head with a decided increase in staining power. Then the sperm head gradually seemed to shorten and assume various irregular and sometimes angular shapes, still retaining its high staining power. Finally these small deeply staining heads seemed to become larger and vesicular, and typical nuclei were formed. Accordingly it would seem that when first formed these vesicles are so delicate that they cannot stand this technique without shrinking all out of shape.

From these experiments we must conclude that in yolk and white of egg the spermatozoön undergoes the transformation into a nucleus. We have not noticed any mitosis or asterformation and we are, therefore, not yet in a position to state that the spermatozoön can undergo mitosis outside the egg.

#### PLATE 1

##### EXPLANATION OF FIGURES

All the figures were drawn with a camera from preparations of chicken spermatozoa and nuclei into which the spermatozoa were transformed.

The vesicles in figs. 1 to 6 all stain more lightly than the substance surrounding them, as is represented in fig. 6. To represent the preparations faithfully figs. 1-5 should also have a background similar to that in fig. 6. All except fig. 4 are from preparations fixed in Flemming's fluid.

1 Chicken spermatozoa showing an early stage in the vesicle formation, and the beginning of the bending of the spermatozoön in the middle-piece region. Sperm kept in white of egg two hours, forty-five minutes. Stained and examined in Herla's malachite green and vesuvin mixture.  $\times 1620$ .

2 Spermatozoa with vesicles as in fig. 1 but more completely bent on themselves in the middle-piece region. All of the loops contain vesicles which are seen exactly from the side in *a* and *c* and slightly from the edge in *b*. From same preparation as fig. 1.  $\times 1620$ .

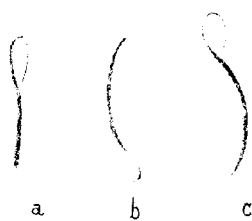
3 Spermatozoa similar to those in fig. 2 but seen edge on. From same preparation as figs. 1 and 2.  $\times 1620$ .

4 Spermatozoön showing the maximum development of the vesicle before the bending of the sperm head begins. Sperm kept in yolk for about four hours. Killed, stained and examined in Herla's.  $\times 640$ .

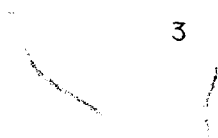
1



2



3



4



## PLATE 2

### EXPLANATION OF FIGURES

5. Sperm heads which have been bent so that they are either within or in contact with the vesicle throughout their entire length. In *b* the flagellum and middle piece of the tail are still visible, but in *a* and *c* these can no longer be made out. Sperm in  $\frac{1}{10}$  Ringer solution + 0.35 per cent  $\frac{1}{10}$  NaHCO<sub>3</sub> for five hours.  $\times 1750$ .

6. Sperm heads contained in vesicles; *a* still showing remains of tails; shows maximum development that the vesicles attain in Ringer solution. Sperm in  $\frac{1}{10}$  Ringer solution four hours. Dark background due to coagulated egg albumen which was mixed with the Ringer solution immediately before fixing to prevent the vesicles being washed off the cover glass. Herla's.  $\times 1620$ .

7. Early stages in the solution and transformation of the sperm head within the vesicle. From same preparation as figs. 1, 2 and 3.

8. Vesicles in which part of the chromatin is present as discrete granules, and part can still be recognized as the portion of the sperm head which adjoins the tail. In *b* two masses of cytoplasm can be seen anteriorly. Herla's.  $\times 1620$ .

9. Sperm heads transformed into nuclei. Chromatin as far as could be determined all next to the membrane. Sperm in egg albumen twenty-three hours. Gentian violet and balsam.  $\times 5000$ .

10. Spermatozoa transformed into nuclei. *a* also appears to have some vacuolated protoplasm. Sperm in egg albumen twenty-six hours. Herla's.  $\times 3100$ .

11. Spermatozoa transformed into nuclei. Chromatin all on nuclear membrane. Gentian violet and balsam.  $\times 3100$ .





## STUDIES IN CYTOLOGY

### I. A FURTHER STUDY OF THE CHROMOSOMES OF TOXOPNEUSTES VARIEGATUS

### II. THE BEHAVIOR OF THE CHROMOSOMES IN ARBACIA-TOXO- PNEUSTES CROSSES

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TWENTY-ONE FIGURES

#### I

In 1910 Miss Heffner made a study of straight fertilized *Toxopneustes* eggs and reached the conclusion that there were two classes of zygotes, one with two and one with three V-shaped chromosomes in each anaphase plate. This condition showed a similarity to the one described by Baltzer ('09) for *Echinus* and was regarded as an indication that its significance was the same, namely that in *Toxopneustes* there were two kinds of unfertilized eggs.

My observations on *Hipponoë* crosses ('11-'12) taken in connection with those of Miss Pinney ('11) on straight fertilized *Hipponoë* eggs led me to believe that possibly there might be another interpretation for *Toxopneustes* and it occurred to me that the facts might be determined most readily by studies of the chromosomes of the egg in chemically fertilized eggs and of the chromosomes of the spermatozoan in fertilized enucleated egg fragments. The question to be decided was whether the unpaired V in zygotes containing three V-shaped chromosomes, was to be associated with the egg or with the spermatozoan.

The work upon which this paper is based was begun in the Laboratory of the Bureau of Fisheries at Beaufort, N. C. I am indebted to the Hon. George M. Boers, Commissioner of Fisheries, for the privilege of working in this laboratory and to Mr. Henry D. Aller, Director of the laboratory, for many courtesies.

*A. Chemically fertilized eggs*

The method of chemical fertilization that I used was that of Loeb ('09), with a very slight modification. The eggs were placed in a butyric acid mixture (6 cc.  $\frac{N}{10}$  butyric acid + 94 cc. sea water), for from one and one-half to two and one-half minutes, transferred from this to sea water to which an amount of  $\frac{N}{10}$  NaOH solution calculated to be sufficient to neutralize the amount of butyric acid which had been carried over in the pipette, had been added; allowed to remain in this for twenty minutes; transferred to hypertonic sea water, (13 cc.  $2\frac{1}{2}$  m. NaCl + 87 cc. sea water), for fifteen to twenty-five minutes; and finally transferred to sea water.

A larger percentage of eggs formed membranes when placed in sea water plus NaOH, upon removal from the butyric acid mixture, than when placed in straight sea water.

With this sea urchin, *Toxopneustes*, it is possible to determine the proper length of time that the eggs should remain in the hypertonic sea water by observing nuclear changes in the egg. Hindle, ('10), in describing the parthenogenetic development of *Strongylocentrotus purpuratus* says, in one place, that no apparent changes "beyond a slight reduction of the clear zone of hyaloplasm surrounding the nucleus" may be observed while the eggs are immersed in the hypertonic solution, and in another place, "during the treatment with hypertonic salt solution there is a slight increase in the size of the nucleus and the clear zone almost disappears."

In *Toxopneustes* the nucleus and the cytoplasm immediately surrounding it may be seen to be in a state of activity. From the surface of the nucleus, processes seem to push out and then retract, the nucleus meantime enlarging in volume, while in the cytoplasm, currents showing a movement of substance toward the nucleus may be seen.

If the eggs be transferred from the hypertonic sea water at the time when, in the greatest number, the nucleus has reached its maximum size, just prior to the bursting of the membrane, it will be found that a larger percentage of regularly segmenting

eggs may be obtained than if the transfer be made at any other time.

It should be remembered that Hindle and I have worked with different eggs and that the processes are not necessarily exactly the same in both. I am inclined to believe, however, that the greater opacity of the *Strongylocentrotus* egg has prevented an observation similar to the one described here.

In practice I found it convenient to divide the eggs into two portions as they were removed from the butyric acid mixture, placing those removed during the interval one and one-half to two minutes in one dish and those removed during the interval two to two and one-half minutes in another. Sometimes one lot, sometimes the other was better. In the work I used finger-bowls of about 400 cc. capacity. Probably the greatest advantage accruing from the use of the NaOH was that of being able to use smaller amounts of sea water and thus concentrating the mass of eggs.

A considerable number of experiments for the determination of the most favorable hypertonic sea water were made. Theoretically it would seem as though a hypertonic solution containing all of the salts of sea water might be more favorable than sea water increased in one salt only. However, neither the hypertonic solutions made by the evaporation of sea water, nor the use of hyper molecular Van't Hoff solutions offered any advantages over the solution mentioned above.

It seems scarcely necessary to note that the experiments were carried out with extreme care. Sterilized dishes and pipettes, and sea water heated to 70° C. cooled and filtered, were used throughout the work. Controls to indicate whether chance fertilization had taken place were kept throughout.

My observations on the external and internal changes in the egg agree, except as above noted, with the descriptions of Loeb, Hindle and Wilson, and therefore need no discussion here.

At first thought it might seem that after the exceedingly careful work that has been done on the cytology of artificially parthenogenetic *Toxopneustes* eggs nothing would be gained by a further study of such eggs. It must be remembered however, that the



work which has been done up to this time has gone no further, with respect to the chromosomes, than the determination of the presence of the haploid number and that in general the chromosomes were rod-like in form.

Within the past five years, partly from the knowledge that in Echinoderms chromosomes of different form are associated with different species and partly from the knowledge of chromosomes showing peculiarity of form in other phyla, the necessity of a further insight into the conditions existing in chemically fertilized eggs has arisen.

This new study of the chromosomes in artificially parthenogenetic eggs has brought out the fact that all of the eggs are alike in that in each anaphase plate there are two V-shaped chromosomes.

It must be mentioned again that in many instances it is extremely difficult to determine the V-shaped elements in the spindle. The arms of the V are frequently in contact with each other, giving the chromosome the appearance of a very thick rod. In some cases it is possible to ascertain that the rod is actually double; in others it becomes a matter of deduction. The conclusions reached are based on the study of a large number of spindles and where it has been necessary to make an interpretation of the nature described, it has been made in accordance with the evidence given by the clearer spindles and not with a preconceived idea of what the conditions should be.

It is noteworthy that the best chances for an exact determination of this form of the chromosomes is afforded by eggs in which the number of asters is above the normal. This is due to the fact that in such eggs the chromosomes are widely scattered.

In fig. 1 *A* and *B* a multipolar spindle with apparently six asters is shown. There are eight V's (four pairs) shown in this egg, three passing to pole *A*, three to pole *B*, one to pole *E* and one to pole *F*. The most probable explanation of the condition shown here lies in the assumption of the doubling in number of the chromosomes by a monaster division, followed by the multipolar divisions shown in the illustration. Such a method of behavior may be seen in many of the living eggs during the early develop-

ment. In addition this conclusion is practically the only one which can be reached in view of the now well established fact of the doubling of the number of chromosomes during monaster formation.

Fig. 2 would be difficult to interpret, were it considered on its own merits alone. The long rod in each anaphase plate is evident. The V's appear here simply as thickened rods; 19 chromosomes are shown in each plate. Fig. 3 shows a similar state of affairs. Fig. 4 is similar with the exception that the V's in 4 A are evident as such.

The description for these figures may be taken as typical of the large number studied in which the chromosomes could be made out clearly. I must also state that in my preparations, representing sixteen very successful chemical fertilizations, the number of abnormal chromosomes is very high. There is nothing like the almost uniformly even and regular series of division figures that may be seen in sections of normally fertilized eggs, nor do the figures approach in regularity those which I obtained from the artificially parthenogenetic starfish egg in 1906. The evidence here seems to be a confirmation of the statement made by Nemec ('11) that the form of the chromosomes may be changed by reagents although the processes of mitosis may be unmodified.

#### *B. Fertilized enucleated egg fragments*

The eggs were broken into fragments by being shaken in a test tube with broken bits of cover glass. For one series this mass was turned out in a flat dish and enucleated fragments picked out under the microscope with a pipette. The fragments were then fertilized with *Toxopneustes* sperm. Fertilization membranes were formed. The tempo of cleavage was the same as in eggs with a nucleus. Other series were prepared by shaking the eggs into fragments as above noted and fertilizing all of the fragments. In these the distinction between fragments with the double number of chromosomes and those with the half number is easily seen. The study of fresh material stained with Schneider's aceto-carmine did not give especially useful results.

The study of the sections of the preserved material was more easily made than was the study of the artificially parthenogenetic material. The evidence from this material seems to show conclusively that there are two classes of spermatozoa with respect to the V-shaped chromosomes, one class with one V, the other with two.

In fig. 5 one V and the long rod are shown. The number of chromosomes in each plate is 18. In fig. 7 two V's and the long rod are evident, 19 chromosomes are present. In fig. 8, one V with the arms separated, one V with the arms in contact and the long rod. Probably 19 chromosomes are present.

## II

While working with the phase of the question just described it occurred to me that possibly conclusive evidence might be given by segmenting eggs from crosses between *Arbacia punctulata*, in which as I showed in 1907, the chromosomes are all small, and *Toxopneustes*, in which the chromosomes are larger.

I succeeded in making this cross for the first time during the past summer ('11). Up to that time I had not been able to secure a usable percentage of fertilizations by either the Hertwig method or the Loeb method, but by combining the two methods I obtained fertilization in a fair number.

The *Arbacia* eggs were allowed to stand in sea water for four hours, then transferred to 400 cc. sea water + 8 cc.  $\frac{N}{10}$  NaOH for five minutes, and then *Toxopneustes* sperm added. Fertilization membranes were formed on from 50 per cent to 90 per cent of the eggs, in different lots.

*Toxopneustes* eggs were allowed to stand in sea water for two hours, then placed in 400 cc. sea water + 6 cc.  $\frac{N}{10}$  NaOH for five minutes and *Arbacia* sperm then added. Fertilization membranes were formed on about 10 per cent of the eggs.

Numerous experiments showed the durations given to be the best. From these fertilizations only a few embryos developed to the pluteus stage, and all of these showed their hybrid origin. The skeleton was of an intermediate type. Most of the embryos

develop very irregularly, pronounced abnormalities appearing during the blastula stage.

The correlation between development and the behavior of the chromosomes is very striking. In a few instances the mitosis of the first segmentation is regular, with very little elimination of chromosomes. In the large majority of instances the behavior of the chromosomes is irregular from the beginning, many of the chromosomes being massed together and eliminated from participation in further mitosis.

It has been suggested that the reason that the chromosomes of one species or the other lag during division in some crosses lies in the differences in normal division rate. It is interesting, in this connection that in the *Arbacia* ♀ × *Toxopneustes* ♂ cross the *Toxopneustes* chromosomes lag although the division rate in this species is more rapid than in *Arbacia*. It is clear that the cause for lagging must be some other than that of difference in division rate. I have previously pointed out that in any *Hipponoe*-*Toxopneustes* cross cleavage is hastened by the *Toxopneustes* sperm, i.e., that of the quicker species.

#### ARBACIA ♀ × TOXOPNEUSTES ♂ CROSS

The chromosomes in the zygotes of *Arbacia punctulata* are in general short and rod-like in form. Owing to their extremely small size I have made no prolonged effort to make out individuality of form. Fig. 6 represents one of three sections passing through a mitotic figure in anaphase. The total number of chromosomes is about forty. There is some variation in length, the longest chromosomes in *Arbacia* exceeding in length the shortest in *Toxopneustes*.

Figs. 10-17 represent anaphases of the first division of the crosses. These represent the most nearly normal figures that were found.

Fig. 10 *A*, *B* and *C* represent the most normal division figure found. Of the *Toxopneustes* chromosomes the long rods and the V's may be recognized. Counting all of the chromosomes present the formula would be  $3\frac{1}{2}$ ; those lagging at the center probably would have failed to be included in the daughter nuclei. Most of

these lagging chromosomes may be seen by their size and form to be *Toxopneustes* chromosomes.

Fig. 11 *A*, *B* and *C*, chromosome formula  $\frac{30}{32}$ . Long rods and *V*'s in anaphase plates; some *Toxopneustes* chromosomes lagging between the plates.

Fig. 12 *A*, *B* and *C*, chromosome formula  $\frac{32}{32}$ . Some of the chromosomes in 12 *C* are probably fragments of those in lower 12 *B*. *Toxopneustes V*'s not present. Apparently there has been an elimination of *Arbacia* chromosomes.

Fig. 13 *A* and *B*, chromosome formula  $\frac{30}{30}$ . One *Toxopneustes V* and the long rod present. Lagging chromosomes probably of *Toxopneustes*. There seems to have been an elimination of *Arbacia* chromosomes.

Fig. 14 *A* and *B*, chromosome formula  $\frac{32}{32}$ . Long *Toxopneustes* rod absent. Some of both *Arbacia* and *Toxopneustes* chromosomes have been eliminated.

Fig. 15 *A* and *B*, chromosome formula  $\frac{32}{32}$ . *Toxopneustes V*'s not present. Full number of *Arbacia* chromosomes does not seem to be present.

Fig. 16 *A*, *B* and *C*, chromosome formula  $\frac{31}{31}$ . *Toxopneustes V*'s not present. There has been an elimination of both *Toxopneustes* and *Arbacia* chromosomes.

Fig. 17 *A*, *B* and *C*, chromosome formula  $\frac{33}{33}$ . Pronounced lagging of *Toxopneustes* chromosomes between daughter plates.

#### THE *TOXOPNEUSTES* ♀ × *ARBACIA* ♂ CROSS

Passing now to the reciprocal cross, figs. 18-21, we notice at once a difference in the appearance of the figures, the number of chromosomes present in each plate being less than in the plates of the reciprocal cross.

Fig. 18 *A* and *B*, chromosome formula  $\frac{29}{31}$ . Both of the *Toxopneustes V*'s and the long rod are present. Most of the *Arbacia* chromosomes have failed to enter the division figure.

Fig. 19 *A* and *B*, chromosome formula  $\frac{29}{31}$ . Both of the *Toxopneustes V*'s and the long rod are present. Nearly all of the *Arbacia* chromosomes have been rejected.

Fig. 20 *A* and *B*, chromosome formula  $\frac{17}{17}$ . Almost a typical *Toxopneustes thelykaryotic* figure. Possibly one or two *Arbacia* chromosomes present.

Fig. 21 *A* and *B*, tripolar figure. Total number of chromosomes present about 85. Four *Toxopneustes* V's (two pairs) present. If this egg was fertilized by two *Arbacia* spermatozoa about half of the *Arbacia* chromosomes have been eliminated.

I have already pointed out the fact that few of the *Arbacia*-*Toxopneustes* hybrids pass through the gastrula stage. From my experiments I have obtained very few plutei indeed, and these few plutei showed evidence of their hybrid origin. If we examine some of the more irregular division figures, not only of the first cleavage but of the later cleavages, we shall find many very pronounced abnormalities; we shall find that not only may the chromosomes of the sperm be involved but that those of the egg as well fail to take their places in the spindle, the result being that the full haploid number of neither parent is present. This fact may well be intimately associated with the abnormal development during the blastula stage.

The only cytological investigation having immediate connection with these crosses is that of Baltzer ('10), on reciprocal *Arbacia pustulosa* crosses. Baltzer has shown that the form of the chromosomes in this species is like those of *Arbacia punctulata* which I described in 1907. Baltzer made six *Arbacia* crosses.

*Strongylocentrotus* ♀ × *Arbacia* ♂

*Sphaerechinus* ♀ × *Arbacia* ♂

*Echinus* ♀ × *Arbacia* ♂

*Arbacia* ♀ × *Strongylocentrotus* ♂

*Arbacia* ♀ × *Sphaerechinus* ♂

*Arbacia* ♀ × *Echinus* ♂

In all of these crosses the results are in general like those which I have just described for *Arbacia punctulata*. In all of the crosses, in most individuals, there is an 'Erkrankung' before or during the blastula stage, this indisposition being so marked as to resemble a sudden poisoning of the embryos.

In Baltzer's *Arbacia* ♀ by *Strongylocentrotus* ♂ or *Echinus* ♂ crosses from 8 to 10 chromosomes were eliminated. In the *Arbacia*-

cia ♀ by *Sphaerechinus* ♂ cross about 18 chromosomes were eliminated (Baltzer '10, text figs. 18 and 19). In Baltzer's *Echinus* ♀, *Strongylocentrotus* ♀ and *Sphaerechinus* ♀ × *Arbacia* ♂ crosses two types of behavior were apparent. In the first two cases elimination of chromosomes occurred during the blastula stage; in the last case the chromosomes were retained. In all of these cases the skeleton of the plutei was nearer the maternal type than the paternal, but the hybrid nature was evident.

My *Arbacia* ♀ × *Toxopneustes* ♂ cross is almost identical in its behavior to Baltzer's *Arbacia* ♀ × *Echinus* ♀ or *Strongylocentrotus* ♂ cross. My *Toxopneustes* ♀ × *Arbacia* ♂ cross is unlike any of Baltzer's *Arbacia* ♂ crosses, in that elimination of chromosomes took place during the cleavage. I shall return to these points later.

#### SUMMARY

The results of this investigation may be summarized as follows:

1. The study of artificially parthenogenetic eggs has shown that the eggs of *Toxopneustes* are all alike in that each contains two V-shaped chromosomes.
2. The study of fertilized enucleated fragments of *Toxopneustes* eggs has shown that *Toxopneustes* spermatozoa are of two classes, one class containing one V-shaped chromosome, the other containing two V-shaped chromosomes.
3. The study of the *Arbacia* ♀ × *Toxopneustes* ♂ cross has shown that elimination of both *Arbacia* and *Toxopneustes* chromosomes may take place during cleavage.
4. The study of the *Toxopneustes* ♀ × *Arbacia* ♂ cross has shown that nearly all of the *Arbacia* chromosomes may be eliminated in the early cleavage.

#### DISCUSSION

The first point requiring consideration is in connection with the results of the study of parthenogenetic eggs and of fertilized enucleated egg fragments of *Toxopneustes*. I have shown here that *Toxopneustes* is in agreement with Hipponoë ('11, '12), in that the

female is **homogametic** and the male digametic rather than with *Strongylocentrotus* (Baltzer, '09), in which the female is digametic.

I still feel that we are not yet able to apply one of the now accepted sex formulae to either *Hipponoë* or *Toxopneustes*. It is futile, at this time, to attempt to make a definite count of the chromosomes in these eggs. I feel reasonably sure that the number in the zygotes is 37 and 38; but granting this to be true, no reliable conclusion can be drawn until we know the facts concerning synapsis in the formation of the germ cells. It is possible that the question can be settled only by the study of the oögenesis and spermatogenesis in these forms, but so much has been determined by the experimental method that one may hope that some Echinoderm may be found which will afford material favorable enough for deciding even this point.

It is interesting in this connection to note that since the females of *Hipponoë* and *Toxopneustes* are homogametic we should obtain uniformly individuals of the same sex from chemically fertilized eggs. The conditions in *Hipponoë* indicate that these individuals would be female.

It must also be noted that my results are not quite in accord with those of Miss Heffner. My observations would give us two classes of *Toxopneustes* zygotes, one with three and one with four V-shaped chromosomes rather than one with two and one with three.

My investigation has shed no further light on the nature of these idiochromosomes; we do not know whether they are compound, whether they are single and have this individual form, or whether the form is due simply to the place of attachment of the spindle fiber. The last idea seems untenable in the light of their definite numerical occurrence.

Turning from these purely cytological considerations to some of the facts concerning heredity in Echinoderms we must first notice that if one wishes to speak with accuracy it is now impossible to make the broad statement that all of the individuals of a given cross are maternal or paternal, or even intermediate, in a strict sense, in character.



We are at a point now where we may gather together the many different accounts that have been given and restore order in a field which has been in a state of disorder. We have conclusive evidence that under certain conditions, most of the embryos of a given cross will have a skeleton of a definite type and we also know that a smaller number may depart radically from this type. Thus we may obtain from eggs of one female fertilized by sperm from one male a complete series of skeletons ranging from the purely maternal to the purely paternal form.

When we compare these series numerically with a similar series of zygotes from some species, in the cleavage and slightly later stages we find the same sort of variation in the number of retained maternal and paternal chromosomes. In other words, there is as great a variation, of its kind, in the kind of chromosomes in cross fertilized eggs as there is in the kind of skeleton in hybrid plutei. This is not true for all species. In some hybrids all of the chromosomes are retained and a dominance of one kind of skeleton over another is exhibited.

The facts regarding the retention or elimination of chromosomes and the character of the ensuing pluteus are of interest. Presented briefly they are:

1. Elimination of no chromosomes and dominance of one species over the other with respect to the character of the skeleton.

*Examples:* *Toxopneustes* ♀ × *Hipponoë* ♂  
*Echinus* ♀ × *Antedon* ♂ (Baltzer)  
*Strongylocentrotus* ♀ × *Antedon* ♂ (Baltzer)

2. Elimination of part of the chromosomes and dominance of one species over the other with respect to the character of the skeleton.

*Examples:* *Hipponoë* ♀ × *Toxopneustes* ♂  
*Echinus* ♀ × *Sphaerechinus* ♂ (Baltzer)  
*Strongylocentrotus* ♀ × *Sphaerechinus* ♂ (Baltzer)

3. Elimination of no chromosomes and skeleton of intermediate character.

*Examples:* *Sphaerechinus* ♀ × *Strongylocentrotus* ♂ (Baltzer)  
*Sphaerechinus* ♀ × *Arbacia* ♂ (Baltzer)

4. Elimination of part of the chromosomes and skeleton of intermediate character.

Examples: *Toxopneustes* ♀ × *Hipponoë* ♂  
*Arbacia* ♀ × *Echinus* ♂ (Baltzer)  
*Arbacia* ♀ × *Toxopneustes* ♂  
*Toxopneustes* ♀ × *Arbacia* ♂

5. Elimination of part of both maternal and paternal chromosomes and inhibition of development.

Examples: *Arbacia* ♀ × *Toxopneustes* ♂  
*Toxopneustes* ♀ × *Arbacia* ♂

This list of examples is not meant to be exhaustive but is given simply as a means of illustration.

*It is evident that in a single series there is a correlation between the types of larvae exhibited and the behavior of the chromosomes. It is further evident, that taking echinoid hybrids as a whole, there is a series of well defined grades, from retention of all chromosomes and preponderance of one type over another, through retention of all chromosomes and a blending of type, to a rejection of more than the half number and failure of development.*

It must be pointed out that such examples as *Echinus* ♀ × *Antedon* ♂ are in a way misleading to one who does not remember that the crinoid larva of early age has no skeleton. We should not expect a spermatozoan having no determinant for a skeleton to produce much effect in an egg having such a determinant or determinants.

It should further be pointed out that such cases as those coming under paragraph 2, e.g., *Hipponoë* ♀ × *Toxopneustes* ♂ as well as the fertilizations of the echinoid egg by the sperm of annelids and molluscs (Godlewski, '11, Kupelwieser, '09), and the fertilization of eggs which have been given a certain impulse to parthenogenetic development by means of chemicals, give as these authors have pointed out, thelykaryotic larvae. Practically larvae derived from such crosses inherit from the egg parent alone just as strictly as if the eggs had been caused to develop from the first by artificial chemical fertilization.

These facts represent a definite advance in our ideas concerning the relation of chromosomes to somatic characters. They do not however aid us in deciding the question as to whether the nucleus is the sole bearer of the determinants of one character or the other. Godlewski ('11), in his expression that neither the nucleus alone, nor the protoplasm alone, but both parts of the cell body are concerned, in the determination of hereditary characters, and that for the development of the paternal characters an interaction between paternal nucleus and protoplasm is indispensable, raises an old objection in a new form and one which it is exceedingly difficult to deny. As our knowledge of the interaction of nucleus and cytoplasm increases, particularly along the lines of nuclear synthesis, we have convincing evidence that the chromatin requires a very definite environment in order that it may increase. Conklin's ('12) observations on the fate of chromosomes centrifuged into the yolk in *Crepidula* is one case in point. It is evident that Godlewski's position is correct in so far as concerns the persistence and growth of the paternal nuclear material, and this material must persist and increase if it is to influence development. How much farther than that we may go in insisting on the necessity of all parts of the paternal germ cell body is somewhat doubtful.

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## PLATE 1

### EXPLANATION OF FIGURES

All of the figures were drawn from sections with the aid of a camera lucida at a magnification of 1500 diameters. They were then doubled in size by means of a drawing camera and compared with the original preparations. The figures have subsequently been reduced one half in publication.

1-4 From chemically fertilized eggs

1A and B Six poled division figure.

2 Lateral view anaphase plate of first cleavage.

3 Lateral view anaphase plate of first cleavage.

4A and B Lateral view anaphase plate of first division.

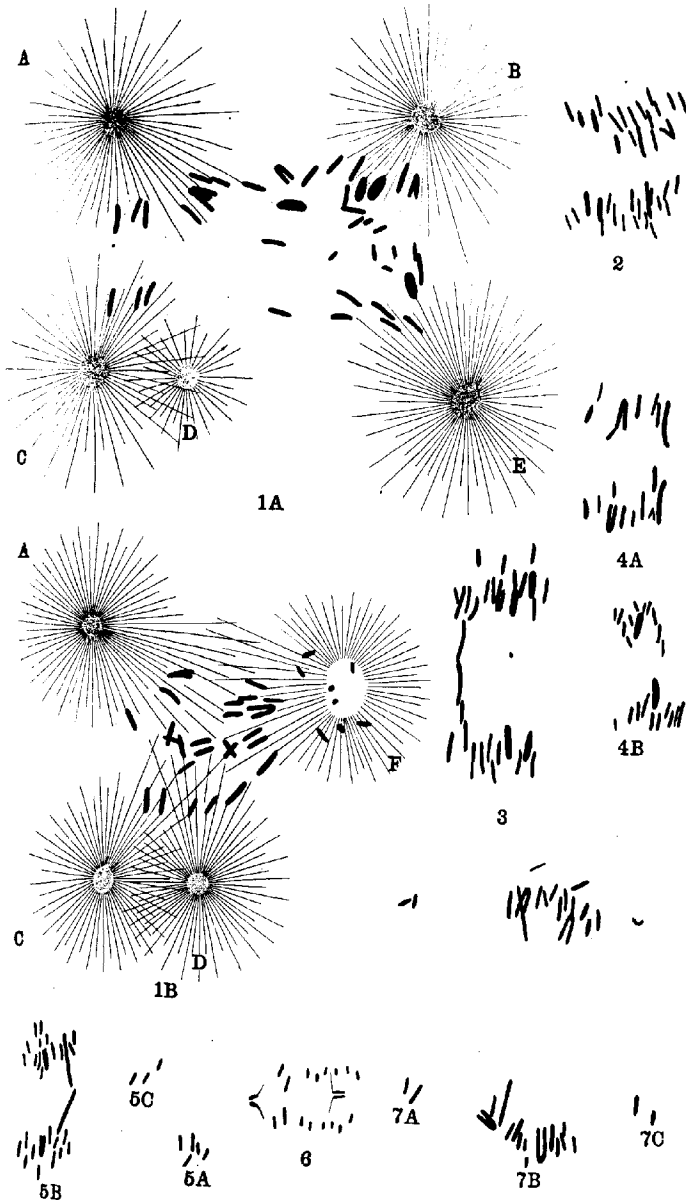
Arbacia

6 Lateral view of one section of an anaphase plate of a straight fertilized Arbacia egg.

From fertilized enucleated egg fragments

5A, B and C Three sections of same mitotic figure in anaphase. One V and one long rod.

7A, B and C Lateral view of anaphase plates as in fig. 5. Two Vs and one long rod.



## PLATE 2

### EXPLANATION OF FIGURES

8A, B and C Lateral view as above. Two Vs and one long rod.

9A and B Lateral view of anaphase plate. Tripolar figure.

Arbacia ♀ × Toxopneustes ♂ cross

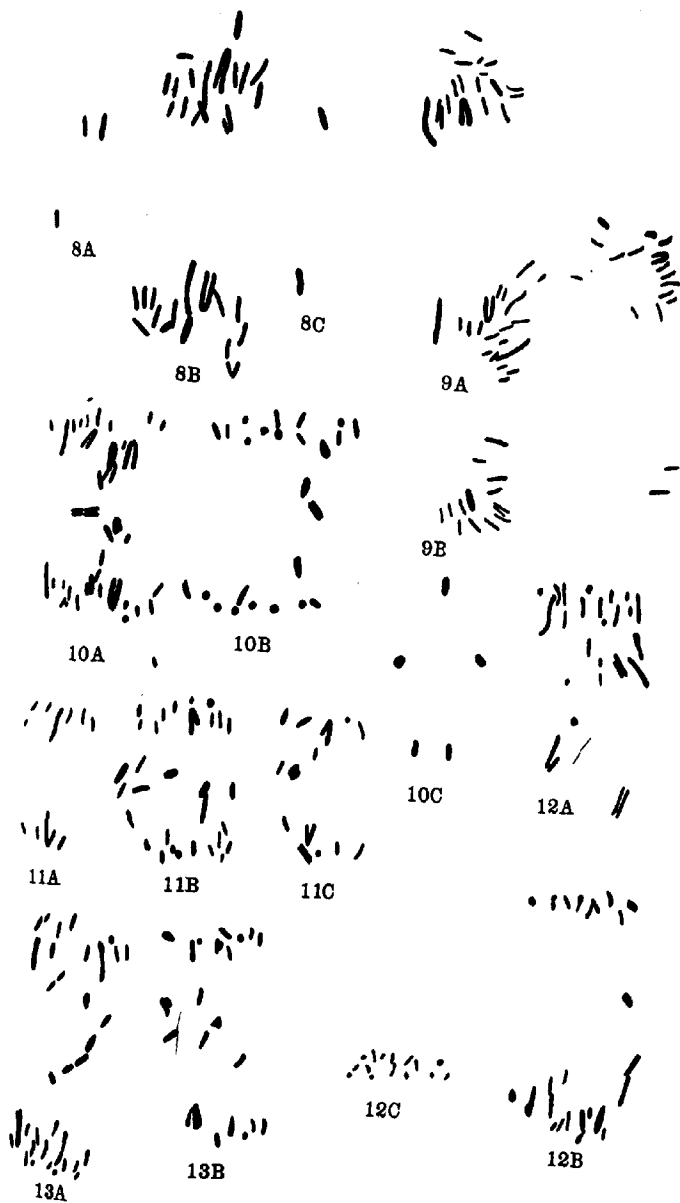
10-17 Lateral views of anaphases of the first division.

10A, B and C The Vs and long rod characteristic of Toxopneustes may be seen.

11A, B and C Long rod and Vs. Some of Toxopneustes chromosomes lagging.

12A, B and C Toxopneustes Vs not present. Probably some of the Arbacia chromosomes have been eliminated.

13A and B One Toxopneustes V and long rod. Lagging and elimination as in fig. 12.





### PLATE 3

#### EXPLANATION OF FIGURES

14A and B No long Toxopneustes rod. Some of both Toxopneustes and Arbacia chromosomes have been eliminated.

15A and B No Toxopneustes Vs. Some of the Arbacia chromosomes seem to be absent.

16A, B and C No Toxopneustes Vs. Some of both Toxopneustes and Arbacia chromosomes have been eliminated.

17A, B and C Pronounced lagging of Toxopneustes chromosomes.

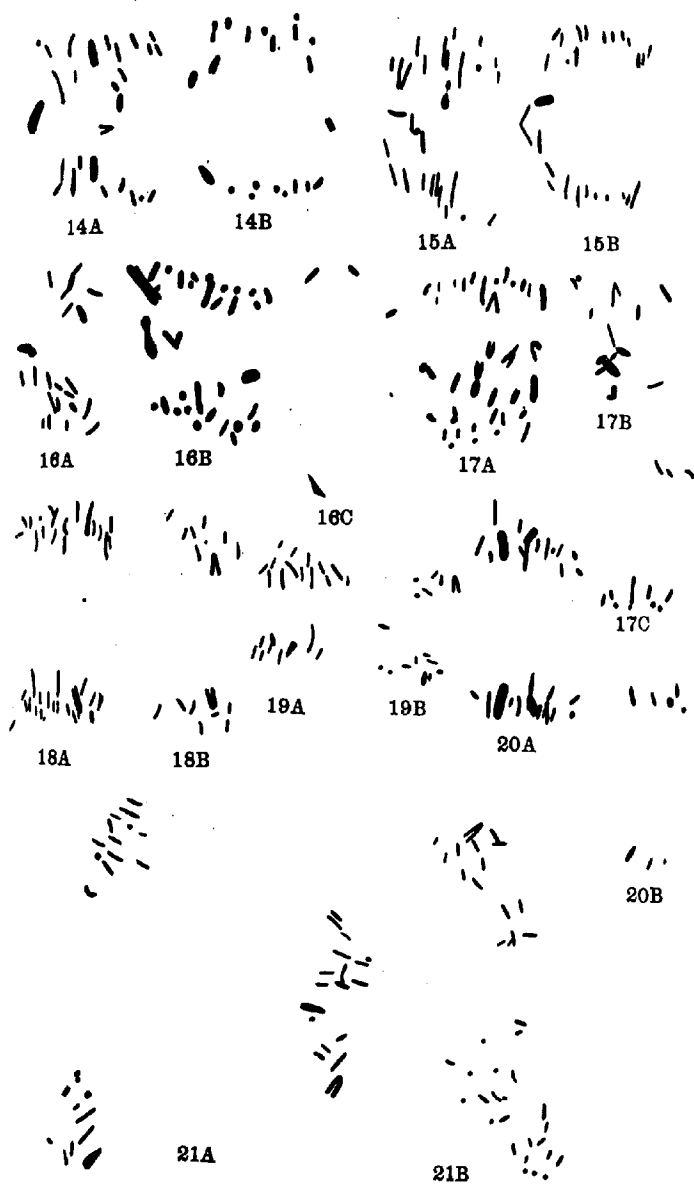
#### Toxopneustes ♀ × Arbacia ♂ cross

18A, B and C Lateral view of anaphase plates. Two Toxopneustes Vs and the long rod. Few Arbacia chromosomes.

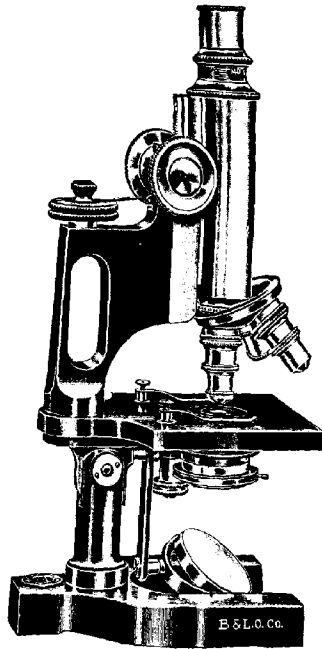
19A and B Lateral view of anaphase plates. Two Toxopneustes Vs and the long rod. Few Arbacia chromosomes.

20A and B. Nearly all of the Arbacia chromosomes are absent.

21A and B Tripolar figure. Two pairs of Toxopneustes Vs are present. Probably about half of the Arbacia chromosomes have been rejected.







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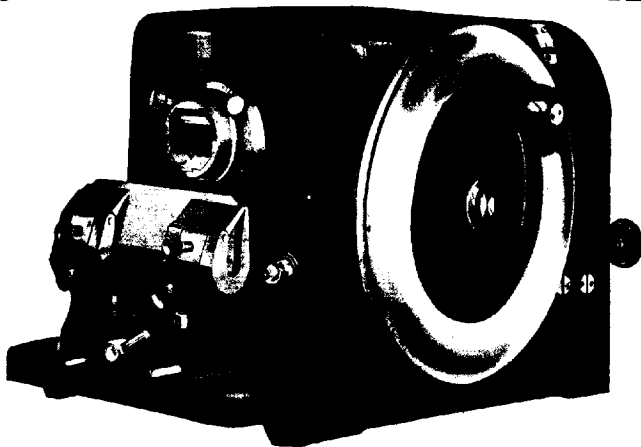
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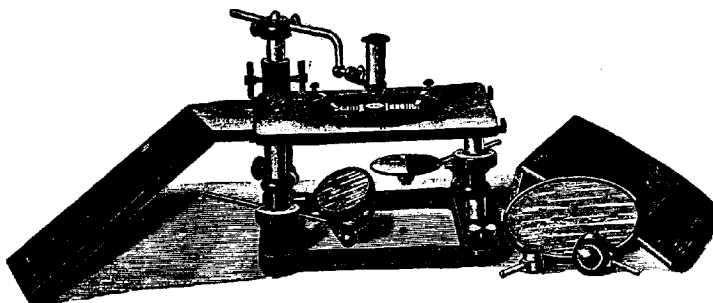
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## STUDIES OF FERTILIZATION IN NEREIS

- III. THE MORPHOLOGY OF THE NORMAL FERTILIZATION OF NEREIS  
IV. THE FERTILIZING POWER OF PORTIONS OF THE SPERMATOOÖN

FRANK R. LILLIE

*From the Marine Biological Laboratory, Wood's Hole, Mass., and the Hull  
Zoological Laboratory, University of Chicago*

FORTY-EIGHT FIGURES

ELEVEN PLATES

In the preceding numbers of these studies dealing with the cortical changes of the egg, and with partial fertilization, reference has been made to certain phases of the normal fertilization. A detailed study was found to be necessary to clear the way for further considerations. The results of this detailed study are presented in the first part of the present paper. The second part deals with the fertilizing power of portions of the spermatoöon and with the theory of fertilization.

### III. THE MORPHOLOGY OF THE NORMAL FERTILIZATION OF NEREIS

#### *1. The spermatoöon: (Fig. 1, a, b, c, d)*

The spermatozoa are unusually large. The head terminates in a very delicate perforatorium which possesses a slight distal enlargement.<sup>1</sup> Between the base of the perforatorium and the chromatin of the head is some differentiated material forming a head-cap (*h. c.*). This is very distinctly differentiated in position and staining reaction from the chromatin of the head. The chromatin of the head is not so condensed as in most spermatozoa;

<sup>1</sup> In the living spermatoöon the perforatorium is shorter and shaped more like the spike of a helmet.



it is often arranged in two masses as shown in fig. 1 with some karyolymph; sometimes the arrangement is more reticular, but the karyolymph is always in evidence in good preparations. The middle-piece is very distinctly set off from the head; it is ring-shaped, very broadly attached to the base of the head. The attachment of the tail is to the *margin* of the ring, and is therefore excentric to the axis of the head. There is a decided asymmetry, particularly illustrated in *b, c, d*, of fig. 1.

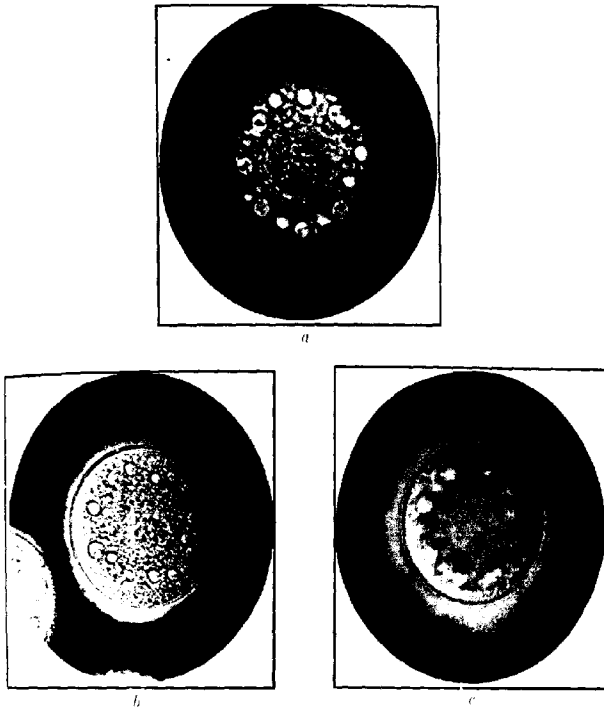
### 2. *The ovum*

The ovum has been described in the preceding parts (Lillie, '11) of this study. A brief description will therefore serve here. It is about  $100\mu$  in greatest diameter, somewhat flattened in a polar direction, and girdled by a double zone of large oil drops embedded in the yolk-bearing protoplasm (text fig. 1). It is bounded by a vitelline membrane and possesses a coarsely alveolar cortical layer about  $7\mu$  in thickness external to the yolk-bearing layer.

### 3. *Observations on fertilization in the living egg*

If the eggs remain unfertilized in the sea-water, maturation does not take place, and the egg remains unchanged with intact germinal vesicle.

*a. The cortical changes.* When insemination takes place a large number of spermatozoa become attached to the ovum if the sperm is present in excess. In about two to three minutes all spermatozoa, with the exception of one, which is alone concerned in the subsequent fertilization, begin to be carried away from the surface of the egg by an outflow of jelly from the ovum. This is more particularly described in the first of these studies (F. R. Lillie, '11). The jelly is formed from the alveolar contents of the cortical layer, which gradually disappears, until in the course of about fifteen minutes the original cortical layer is represented only by the perivitelline space and the delicate walls of the original alveoli crossing this space to the plasma membrane (see study 1).



Text fig. 1. Three photographs of eggs of *Nereis* in ink; *a*, before insemination; *b*, three minutes after insemination; *c*, twelve minutes after insemination. *a* and *b* were taken with direct sunlight and short exposure (one second). *c* with diffuse light and longer exposure (ten seconds). See text for description.

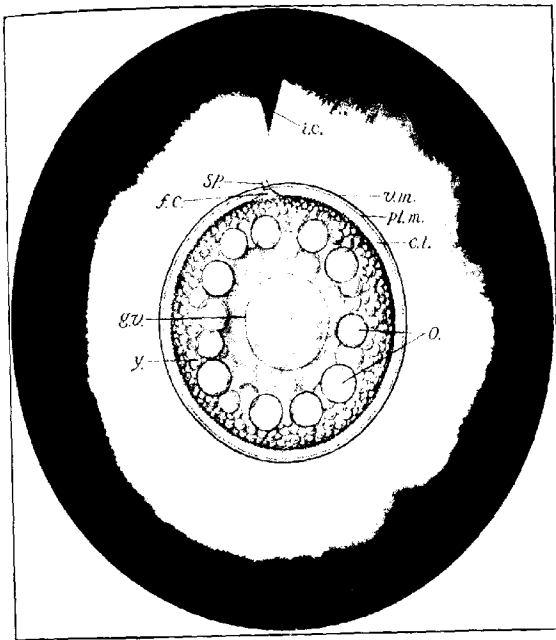
The formation of the jelly may be most readily observed if the eggs are inseminated in sea-water, into which India-ink has been ground so as to make a black suspension. I here reproduce three photographs showing the formation of the jelly under such circumstances (text fig. 1, *a*, *b*, *c*); *a* is an egg taken before insemination; the germinal vesicle and the oil globules stand out very distinctly; the cortical layer may be seen on the left side of the figure, but it does not come out very distinctly because it is seen through a layer of ink between the cover slip, which touches the

top of the egg, and the equator of the egg. Fig. 1 *b* shows the amount of jelly formed about three minutes after insemination; the cortical layer is already much reduced. Fig. 1 *c* was taken twelve minutes after insemination; it shows a great increase in amount of jelly due not only to continued secretion from the egg, but also, presumably, to swelling of the jelly already formed. The walls of the alveoli which contained the jelly-forming spherules may be seen on the right side of this photograph crossing the perivitelline space to the plasma membrane. On the upper side may be seen the fertilization cone and external to it outside the vitelline membrane the spermatozoön. A conical pointer of ink is directed to the spermatozoön. This forms regularly in eggs inseminated in ink and is due to the ink particles pressing in along the tail of the spermatozoön embedded in the jelly, owing to their Brownian movements. This ink cone remains even after the spermatozoön has penetrated and thus serves as indicator of the point of entrance of the spermatozoön up to the time of cleavage. A study of the relation of the point of entrance to the first cleavage plane by one of my students is now under way (see Just, '12).

Text fig. 2 is a drawing from the living egg based on such a photograph as 1 *c*. The details are all clearly shown here and are described in the legend of the figure.

*b. The fertilization cone.* The spermatozoön, that remains after the first jelly-formation, is attached by its perforatorium to the vitelline membrane. The protoplasm of the ovum immediately beneath now forms a conical elevation (fertilization cone) which crosses the perivitelline space and becomes attached to the vitelline membrane beneath the spermatozoön (text figs. 1 *c* and 2). The fertilization cone then gradually retracts and disappears, drawing the vitelline membrane with it so as to form a depression in which the spermatozoön is included. At this stage one may easily imagine that the spermatozoön has been taken into the egg, as it is apt to be concealed in the depression of the membrane; but this is not the case. The stage of greatest development of the depression, corresponding to the complete retraction of the fertilization cone, is about twenty-two to twenty-five minutes after insemination (see figs., Lillie '11, p. 369).

The fertilization cone is no longer seen in the living egg. But the sections show that its substance has become so modified as to form a definite cell-organ, which can be followed for some time after penetration of the spermatozoon.



Text fig. 2. Egg of *Nereis* fifteen minutes after insemination in ink. The black outer circle represents the ink, the clear area between this and the egg is the jelly; the thin dark line is the cortical layer showing the radiating lines of the emptied alveoli; *f.c.*, fertilization cone; *g.v.*, germinal vesicle, drawn perhaps a little too strongly; it begins to break down about this time; *i.c.*, ink cone; *o.*, oil drops; *pl.m.*, plasma membrane; *s.p.*, spermatozoon; *v.m.*, vitelline membrane; *y.*, yolk spherules.

*c. Penetration of the spermatozoon.* Following the development of the depression in the vitelline membrane consequent upon the retraction of the fertilization cone, the perivitelline space

narrows around the entire egg, the depression in the vitelline membrane therefore disappears and the spermatozoön again becomes prominent externally. It remains external until about forty to fifty minutes from the time of insemination and then disappears rather abruptly within the egg. Its penetration coincides with the late anaphase or telophase of the first maturation division, or with the extrusion of the first polar body.

A definite granule to which the tail is attached always remains on the membrane at the point of penetration. The cytological study shows this to be the middle piece, which does not enter the egg. Not only have I repeated this observation frequently in two successive seasons, but it has been demonstrated to classes of students at Woods Hole and to some of the investigators there. *The middle piece and tail of the spermatozoön do not enter in the fertilization of the egg of Nereis.* In view of the emphasis which has recently been put on cytoplasmic contributions by the spermatozoön in fertilization, I paid particular attention to this point in the summer of 1911, and was able to determine not only that it fails to enter at the same time as the sperm-head, but that it can be demonstrated external to the membrane up to the time of the first cleavage at least (cf. also Just, '12).

I have seen the penetration of the spermatozoön take place in all positions from the animal to the vegetative pole, and I cannot say that there is any preferred point of entrance with reference to the poles. The existence of polyspermy, which is not infrequent, proves that there is no preferred meridian for penetration. So it would appear that any point on the surface of the ovum may be used for penetration.

#### 4. *The phenomena of fertilization as seen in sections*

*a Before penetration.* Technique: The eggs are fixed for an hour in Meves' modification of Flemming's fluid made as follows: Chromic acid 0.5 per cent, 15 cc.; osmic acid, 2 per cent, 3.5 cc.; glacial acetic acid, three drops. Staining in iron haematoxylin alone.

The spermatozoön is readily found in sections prior to penetration, and all parts can frequently be recognized, though the tail is usually invisible owing to extraction of the stain. The middle piece is always distinctly differentiated, but it is rather variable in appearance. Its ring-form is usually distinguishable in sections and in some cases one or more minute granules may be distinguished in it. The head stains solid black in the iron haematoxylin. Fifteen minutes after insemination (fig. 2, *a, b, c*) the perforatorium has penetrated the vitelline membrane and is in contact with the fertilization cone.

The fertilization cone itself presents a rather remarkable appearance, and it deserves very careful attention, owing to the rôle that it plays in the penetration of the spermatozoön. Fifteen minutes after insemination it projects somewhat above the contour of the egg, and its substance, which is practically homogeneous, stains much more deeply in the iron haematoxylin than the neighboring cytoplasm. This difference in staining reaction so suddenly acquired indicates a modification of its substance which might be conceived as due to slight coagulation by a fluid introduced by the spermatozoön. In any event the change in stain is due to its relation to the spermatozoön, and it is the first indication of its conversion into a specialized cell-organ.

A fertilization cone has been described in various eggs (echinids, asteroidea, etc.), but in all cases previously described its significance has been, apparently, merely temporary and local, a reaction of the ovum to the spermatozoön with no definable function of its own. The case of *Nereis*, however, is very different, as will be seen from the following description.

At twenty-seven minutes after insemination the fertilization cone no longer forms any projection (fig. 3); it is usually, on the contrary, somewhat depressed in the center. The perforatorium stains somewhat more strongly than before.

At thirty-four minutes after insemination the perforatorium has entered the surface of the fertilization cone to a slight extent (fig. 4), and a small granule appears at its tip, surrounded by a slight clear area. A little later (fig. 5, *a, b, c, d*, thirty-seven minutes after insemination) there is a group of three or four black

granules, which we shall name *attachment granules*, at the tip of the perforatorium embedded near the surface of the fertilization cone. In one case (fig. 5 a) the perforatorium is seen to be double, a condition that I have also observed once in a preparation of spermatozoa alone. The perforatorium now stains very strongly.

The spermatozoön is now, therefore, actually anchored in the substance of the fertilization cone by the perforatorium and attachment granules noted above. I interpret the increased strength of stain in the perforatorium as due to the flow of substance through it from the spermatozoön to form the granules found at its tip in the fertilization cone.

One can give only a more or less probable interpretation of these phenomena. The extreme delicacy of the perforatorium and the presence of a distal enlargement on it make it improbable that it bores through the membrane in a merely mechanical way; and the improbability is strengthened by the fact that the spermatozoön is absolutely immobile after attachment to the membrane. It seems *probable*, therefore, that the vitelline membrane is weakened at the point of application of the perforatorium, presumably by a fluid flowing through the perforatorium, though on account of its extreme delicacy it is impossible to be certain that the perforatorium is tubular. The staining reaction and differentiation of the fertilization cone could then be explained by the assumption that it is affected by a fluid furnished by the spermatozoön, and the appearance of the granules within the fertilization cone as due to inflow from the spermatozoön through the perforatorium. The mechanism of the spermatozoön certainly appears more complex than we have hitherto suspected. I would assume, therefore, that the material of the head cap, forming a reservoir of material at the base of the perforatorium, functions in relation to penetration.

*b. Penetration.* The actual penetration of the head of the spermatozoön is shown in figs. 6, 7 and 8, all taken from material preserved forty-eight and one-half minutes after insemination. The complex made up of the fertilization cone and the head of the spermatozoön acts as a unit. The cone retreats into the interior of the protoplasm and the head of the spermatozoön becomes

a narrow chromatin band as it enters through the minute aperture in the vitelline membrane. Figs. 9 *a* and 9 *b* (fifty-four minutes after insemination) show penetration completed and it will be noted that the middle piece remains external.

Examining now the details of this process, every stage of which is found in the preparations, we may note: (1) The sperm nucleus enters through an aperture in the membrane much smaller than itself, and is hence drawn out into a strand. (2) The inner end of the entering sperm nucleus begins to swell, by absorption of fluid soon after its entrance has begun (figs. 6, 7, 8). (3) Striae appear in the protoplasm between the fertilization cone and the surface of the egg. (4) When penetration has actually begun (fig. 6) the distance, corresponding to the length of the perforatorium, which originally separated the chromatin of the spermatozoon from the attachment granules in the fertilization cone has become very much reduced. The sperm nucleus is now almost or actually in contact with the fertilization cone (figs. 6, 7, 8).

If we inquire into the mechanism of this process, it is quite obvious that the initiative, so to speak, is on the side of the ovum. It is inconceivable that the spermatozoon should be the *primum movens* and push the fertilization cone before it into the protoplasm without being coiled up or bent within the protoplasm. But, in the many instances observed, it is invariably as straight as shown in the figures. The fertilization cone indeed *appears* to actively penetrate, or to be engulfed by the egg protoplasm, and to draw the sperm nucleus after it through the narrow aperture in the vitelline membrane.

The final stage of penetration is shown in figs. 9 *a* and 9 *b*. The entire sperm nucleus is now within the egg cytoplasm, some distance from the periphery, and it will be seen that the middle piece has remained without on the egg membrane. This is perfectly characteristic, if not invariable, and the same observation was made repeatedly on the living egg as already stated. The picture is perfectly clear as shown under a very high magnification in fig. 9 *b*, and not only is the middle piece plainly external, but there is no sign whatever of any sperm-component at the base



of the sperm head. An assumption that the spermatozoön introduces any differentiated structure at the base of the head could, in this case, be due only to a preconception in its favor.

*c. Revolution of the sperm-nucleus and origin of the sperm-aster.* The original orientation of the sperm nucleus within the egg is perfectly apparent, owing to the association of its apex with the fertilization cone from the very beginning of penetration. The whole complex of fertilization cone and sperm nucleus now rotates 180 degrees, so that the fertilization cone, which was placed centrally to the sperm nucleus, comes to lie peripheral to it. In fig. 10 *a* (fifty-four minutes after insemination) the revolution is shown nearly completed. Fig. 10 *b* shows the cone-nucleus-aster complex of the same egg more highly magnified; and figs. 10 *c* and 10 *d* show the entrance point of the spermatozoön involved, with tail (10 *c*) and middle piece (10 *d*) external to the vitelline membrane. The sperm aster is beginning to form in this stage opposite to the fertilization cone, thus in the position of the original middle piece, which, as we have seen, was left on the egg membrane. A minute granule, actually in the substance of the nucleus (fig. 10 *b*) is the point of focus of the rays. In fig. 11 (sixty-four minutes after insemination) this granule, now more clearly recognizable, is still in contact with the sperm nucleus. The rotation of the sperm nucleus is invariable, and no exception has been seen in the numerous cases observed; the point of origin of the sperm aster is just as invariable.

The fact that the sperm aster arises from the base of the sperm nucleus as it is oriented in the spermatozoön, not only in *Nereis* but in all other forms accurately determined, is unquestionably of fundamental significance. I may, however, be permitted to point out that the usual interpretation, which connects the sperm aster with the centrosome of the spermatid (occupying this position in the spermatozoön) is not the only possible interpretation. It is important to emphasize this point because such an interpretation ascribes the fertilizing power of the spermatozoön in the last analysis, not to the spermatozoön as a whole, but to a minute part of it, the centrosome; and it involves a whole theory not only of fertilization, but of cell division in general, and much of cellular

physiology, by ascribing certain very general functions and powers to this minute cell element.

The alternative interpretation, which I would present, is that the appearance of the sperm aster in a position definitely oriented with reference to the sperm nucleus may be a nucleo-plasmic reaction localized by polarity of the nucleus.

In any case, if the centrosome theory is to be retained for *Nereis*, it becomes necessary to assume that, in this form, the centrosome is contained, not in the middle piece, but in the nucleus of the spermatozoön. In the experimental study, which forms the second part of the present paper and study number 4 of the series, I shall present crucial evidence against this assumption; discussion of the matter may therefore be postponed.

After the appearance of the sperm aster, the nucleus penetrates yet more deeply within the egg and leaves the fertilization cone behind near the inner margin of the yolk (fig. 11), where it soon disappears. So far as I know, it is a perfectly unique cell organ, except in its earliest stages, and it is certainly surprising to find it in so highly differentiated a condition, and with apparently so active a function, in the penetration of the spermatozoön, as it is in *Nereis*.

*d. Division of the sperm aster.* The sperm nucleus moves towards the center of the egg, and the aster separates from it a little and divides, forming an amphiaster. A secondary spindle then arises between one or both of the sperm-centrosomes and the inner centrosome of the second maturation spindle (figs. 12 and 13). This has been very fully described by Bonnevie ('10). Shortly after the sperm amphiaster is formed, it is noticeable that one of the asters and centrosomes, is decidedly larger than the other (figs. 13 and 14*a*). Bonnevie has also called attention to this fact, but has not, as I believe, sufficiently emphasized the fact that it is a secondary condition. I must dissent a little from her view that the sperm aster does not always divide prior to the completion of the second maturation division; in my material, at least, the amphiaster is invariably formed prior to this time.

*e. The germ nuclei and origin of the cleavage centers.* After the second polar body is formed, the egg aster gradually dwindles and

disappears (figs. 14 *a*, 14 *b* and 15). There can be not the least doubt of this fact in my material, though Bonnevie, working on the same form, but with different cytological methods, remains doubtful on this point. I shall not attempt an elaborate cytological analysis of the matter here (though the evidence from this side is, I believe, conclusive), because in eggs from which the sperm nucleus has been removed experimentally the gradual disappearance of the egg aster can be traced with complete certainty, owing to the absence of any possible confusion with the sperm aster (see second part of this paper, p. 440). The fully formed egg nucleus in such eggs never has a trace of the aster (see studies 2 and 4). The chromosomes of the egg swell and form vesicles (figs. 14 *a*, 14 *b*, 15), which gradually fuse together and establish the egg nucleus. At the same time the sperm nucleus begins to enlarge (fig. 14 *b*) and the sperm amphiaster becomes less distinct than before and its radiations less extensive (figs. 13, 14 and 15). The sperm amphiaster continues to wane and when the two germ nuclei have come together, the smaller aster has become indistinguishable (fig. 15). The larger aster can, however, always be distinguished through the stages of the germ nuclei, and can be seen to become the larger aster of the first cleavage spindle after the partition wall between the germ nuclei has disappeared (figs. 16 *a*, 16 *b*, 17). At this time a much smaller aster arises opposite the larger (fig. 17), like it in the plane of apposition of the germ nuclei. The first cleavage spindle is thus heterodynamic from its inception, and the first cleavage of the egg is strikingly unequal, as is well known from Wilson's study (Wilson, '94).

In the preceding paragraph I have summarized a very complicated period of the fertilization process to which I have given much study, and which exhibits many interesting cytological details, as for instance, the accumulation of granules in the neighborhood of the germ nuclei (figs. 15 and 16 *a*) which almost certainly escape from the latter (fig. 16 *a*). As regards the main point, the origin of the cleavage centers, there can be no doubt that the larger one is derived from the larger aster of the sperm amphiaster, for it can be followed continuously and is

never absent. The simplest interpretation of the smaller cleavage center is that it represents the smaller sperm aster, although there is a brief period when it is not demonstrable, owing (possibly) to defect in the cytological technique. It is my opinion, then, that the sperm amphiaster becomes the cleavage amphiaster in *Nereis*, as in so many other animals.

### 5. Discussion

If any apology is needed for presenting so strictly a morphological study of such an apparently threadbare subject as the fertilization of the ovum, I might say that the impulse to make it came from an experimental study, and that it is necessary to the experimental results which follow. It has, moreover, yielded some details of observation which deserve to go on record on their own account.

The conclusion that the cleavage centers arise from the sperm centers is in agreement with many other studies. But I am unable to accept the usual conclusion that the sperm centers arise around a centrosome introduced by the spermatozoön into the egg, and that, therefore, the sperm centrosome is the fertilizing agent of the spermatozoön, and the sperm nucleus concerned exclusively with amphimixis. The crux of the problem is precisely here on the question of the origin of the sperm centers. The fact that the middle piece of the spermatozoön which usually includes the spermatid centrosome does not penetrate the egg in *Nereis* is evidence of a certain amount of value only. Defects in cytological technique may always be invoked to explain failure to observe the introduction of a centrosome by the spermatozoön. Little as I may be inclined to admit this, it is necessary to grant some force to this objection where such delicate cytological details are involved. It would, however, I believe, be recognized as crucial evidence that the sperm centrosome is not necessary to fertilization, if a distal fraction of the sperm head alone were proved to form a sperm aster, a certain portion of the base of the sperm head as well as the middle piece being prevented from entering. Such results are described in the second part of this paper.

There has been much discussion during the past year concerning a cytoplasmic basis of certain aspects of inheritance, and a consequent re-investigation of the penetration of the spermatozoön. Following Meves' study ('11) of the rôle of plastochondria in the fertilization of *Ascaris*, in which he concluded that "the plastosomes represent the hereditary substance of the cytoplasm as the chromatin does that of the nucleus," a number of authors investigated the penetration of the spermatozoön in echinids. Dantan ('11) asserts that in *Paracentrotus lividus* and *Psammechinus miliaris*, the entire spermatozoön enters the egg, and he concludes that fertilization should be defined as the union of two complete gametes which fuse nucleus to nucleus and cytoplasm to cytoplasm. Witschi ('11) describes a case in *Strongylocentrotus* in which the tail of the spermatozoön entered in fertilization, but he thinks it probable that in this form the tail is oftener left on the membrane. Ries ('11) describes a curious shedding of an involucre in the penetration of the spermatozoön in *Strongylocentrotus*, but he believes that the axial structures of head, middle piece and tail enter. His account must, however, be accepted with considerable reserve until confirmed. Finally, Meves ('11a) has studied the relation of the middle piece in the fertilization of *Parechinus miliaris*, and believes as the result of his observations that it probably furnishes plastochondria. He says nothing about penetration of the tail, so it is fair to assume that it does not occur in *Parechinus*.

The classical accounts of the penetration of the spermatozoön in sea-urchins, according to which the tail is left on the membrane and only head and middle piece enter seem to be on the whole confirmed so far as the main principle (i.e., the non-essential character of the tail in fertilization) is concerned, since both entrance and non-entrance have been observed. The tail cannot therefore be regarded as supplying a cytoplasmic basis for inheritance in sea-urchins.

I have shown that in *Nereis* the middle piece of the spermatozoön is likewise left on the membrane, so we cannot look to it as a cytoplasmic basis for inheritance in this form as Meves does in the sea-urchin. On the other hand it is possible that the fixation

granules introduced by the spermatozoön represent a cytoplasmic element (whether concerned in inheritance or not), but of this we cannot be certain until their derivation is better known.

The only characteristic thing about the cytoplasmic elements introduced by the spermatozoön is their great variability as to quantity and character in different animals. In *Ascaris* a very large quantity of cytoplasm containing characteristic plastosomes is introduced, as Meves has shown. In many, probably most, forms with flagellated spermatozoa, the entire spermatozoön enters; in some echinids the tail is left without, and in *Nereis* both tail and middle piece fail to enter; and turning to plants, in phanerogams apparently nothing but the nucleus is eventually concerned. There is nothing on the cytoplasmic side to correspond with the regularity of the nuclear phenomena in both animals and plants. In such precise phenomena as those of inheritance a mechanism of equal precision is to be expected, and it must be admitted that on the cytoplasmic side no such mechanism has been discovered. Moreover, as the laws of inheritance are the same for animals and plants, a similar mechanism must exist for both, and such has been discovered only in the nuclei of the gametes. There is bad logic in the assumption that whatever parts of the spermatozoön enter the egg are necessarily concerned in the mechanism of transmission in inheritance, and the view that the cytoplasmic elements of the male gamete are concerned primarily in accessory functions of fertilization, such as locomotion and penetration, is still logically well founded.

#### IV. THE FERTILIZING POWER OF PORTIONS OF THE SPERMATOZOÖN

##### 1. *Introduction and methods*

In the second of these studies (F. R. Lillie, '11) it was shown that the stimulus of the spermatozoön in fertilization involves two phases: (1) an external phase, prior to entrance of the spermatozoön but after its attachment to the egg, in which certain cortical changes are induced, jelly is secreted by the egg and the mechanism of maturation of the ovum is released; and (2) an internal phase beginning after the entrance of the spermatozoön,

which is necessary if cleavage of the ovum is to take place. The present study is a contribution to the analysis of the latter phase based on observations concerning the fertilizing power of portions of the spermatozoön.

It is questionable whether any direct and universal method for such an experiment could be devised, for one would have to overcome the difficulties of isolating a spermatozoön, of operating on it, and of ensuring the entrance of a part into the ovum, under precautions that would preclude the possibility of fertilization by an intact spermatozoön. These difficulties might be overcome by an instrument sufficiently delicate to enable one to amputate parts of the attached spermatozoön before its entrance into the egg. The same result has been obtained in *Nereis* by a method that enables one to operate in bulk, to remove fractions of the attached sperm head of varying size, to observe the entrance of the part remaining attached to the egg, and to study its fertilizing effect, at least to a certain extent.

In brief, the method consists in centrifuging the inseminated eggs of *Nereis* at five minute intervals before penetration and preserving the centrifuged eggs at appropriate times. The effectiveness of the method depends on conditions already described (see part 1 of the present paper, study 3 of the series) which may be summarized briefly as follows: The spermatozoön remains external to the vitelline membrane with its perforatorium embedded in the entrance cone for about fifty minutes, more or less, depending on the temperature, after insemination. It is embedded in the thick viscous jelly secreted by the egg (text figs. 1 and 2). If now, a quantity of eggs be centrifuged with sufficient force, they first accumulate at the distal ends of the tubes in a mass which becomes closely packed together. The jelly, which is of less specific gravity than the eggs, then separates from the latter and forms a layer above the eggs. In squeezing through the narrow interstices between the closely packed eggs the jelly rubs over the surface of each egg and in many cases carries the attached spermatozoön away with it, leaving, however, the perforatorium and attachment granules in the cone as evidence of its former presence. In other cases, especially if the eggs be centrifuged shortly before the time of penetration of the spermatozoön, it

draws out the substance of the head of the spermatozoön, which is very ductile at this time, into a strand, and in numerous cases it carries away the tail and middle piece or variable portions of the head in addition. Partial sperm heads of all sizes are therefore left attached to the egg by the perforatorium. Such partial sperm heads then penetrate, if the eggs be left to develop in seawater, and their behavior may be studied.

In each experiment 8 or 9 stages in the process of fertilization were centrifuged at five minute intervals, in order to be sure that the entire period of penetration of the spermatozoön was covered, because particularly striking results were to be expected from removal of the external part of the spermatozoön after a certain amount had entered (figs. 6 to 8). Several hundred eggs were centrifuged each time. One has to be sure that neither too little or too much centrifuging is done, and it was only as a result of considerable experience extending over three years that sixty revolutions of the handle of the centrifuge in about forty seconds, giving 7200 revolutions of the tubes at a radius of 6 cm., was selected as most favorable.

The following protocol of an experiment will show exactly how the experiments are carried out and the material secured for examination.

## EXPERIMENT 16

September 21, 1911

To preserve a series immediately after centrifuging and thirty minutes later  
Eggs fertilized at 8:28 A.M. Temperature of air, 18°C.; of water, 18°C.

DESIGNATION	CENTRIFUGING	PRESERVATION				OBSERVATIONS ON PERCENT OF SEGMENTED EGGS AMONG THOSE REMAINING	
		a. m.		a. m.			a. m.
16.1	× 60	8:58	16.1.1	8:59	16.1.2	9:29	35 per cent
16.2	× 60	9:03	16.2.1	9:04	16.2.2	9:34	15 per cent
16.3	× 60	9:08	16.3.1	9:09	16.3.2	9:39	10 per cent
16.4	× 60	9:13	16.4.1	9:14	16.4.2	9:42	5 per cent
16.5	× 60	9:18	16.5.1	9:19	16.5.2	9:49	25-30 per cent
16.6	× 60	9:23	16.6.1	9:24	16.6.2	9:53	65 per cent
16.7	× 60	9:28	16.7.1	9:29	16.7.2	9:58	75 per cent
16.8	× 60	9:33	16.8.1	9:34.5	16.8.2	11:03	85 per cent
16.9	× 60	9:38	16.9.1	9:39.5	16.9.2	11:09.5	90+ per cent
Controls—preserved at 8:57, 9:12.5, 9:35 A.M.							90+ per cent
							11.05



The eggs preserved immediately after centrifuging enable one to study the immediate effects of the separation of the jelly on the spermatozoön, and those preserved later show what portions of spermatozoa remaining attached after centrifuging have entered the egg and what their fertilizing power has been up to the time of preservation. Some of the eggs were kept living for estimation of the per cent of eggs that undergo segmentation (last column). In other experiments the eggs were preserved at different periods following centrifuging, because no single experiment gives a sufficient quantity of material for preserving a complete series of stages. In this way from a considerable number of experiments a very complete set of stages was secured.

Each lot of eggs preserved included several hundred which were embedded together in paraffine and cut in serial sections which would usually cover four or five slides under a 50 by 25 mm. cover, if all the material were cut. On one such slide I estimated by counting that there were 374 eggs present; and the 1911 material alone made 376 slides. Only about a fourth of the slides contain the desired stages, and the figures are given only to show that a large quantity of material has actually been under review to give the results. However, I may say that figs. 18 to 23 are all from a single slide, and other interesting stages occurred on the same slide; it is possible in fact to demonstrate the whole set of phenomena from a few slides of the large number prepared.

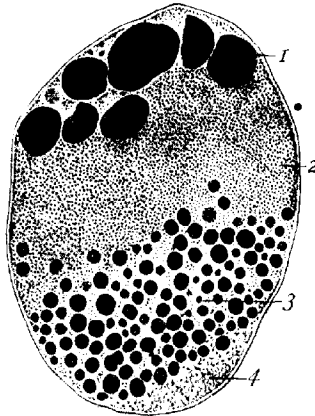
The clew to the whole set of phenomena was given by the discovery of very minute sperm nuclei in the material used for the second of these studies (F. R. Lillie, '11). In the effort to discover the origin of these minute, and presumably partial, sperm nuclei, the whole history was gradually worked out as given here.

The centrifugal force employed causes a very complete segregation of the oil and granules of the egg into four zones which are illustrated in text fig. 3, taken from a section fixed in Meves' modification of Flemming's fluid and stained in iron haematoxylin. These zones are, beginning with the constituents of least specific gravity; (1) the zone of the oil drops; (2) the hyaline zone in which the smaller basophile granules gather for the most part; (3) the zone of the yolk-spheres; (4) a zone consisting of small hyaline

spheres not stained by the osmic acid or haematoxylin, interspersed with basophile granules larger than those of zone 2. The penetration and rotation phenomena appear in the hyaline zone even more clearly than in the normal egg, hence most of the figures illustrating these phenomena are taken from the hyaline zone.

## 2. *The effects on the spermatozoa*

a. *Before the beginning of penetration.* The effects of the removal of the jelly on the spermatozoon have been studied especially on stages shortly before penetration begins, both because these are the stages most affected as shown by the effects on cleavage



Text fig. 3 The zones of the centrifuged egg of *Nereis*

(stages 16.3 and 16.4, in the preceding table) and also because spermatozoa injured at this time would presumably have a better chance to enter, being so near to the actual time of penetration.

Figs. 18 to 23 show successive degrees of injury to spermatozoa from a single experiment (6.5 of 1911). The eggs were preserved immediately after centrifuging 7200 revolutions at a radius of 6 cm. in thirty-five seconds, fifty minutes after insemination, on June 19, 1911, when the temperature of the water was still quite low and the processes correspondingly slow. In fig. 18 the entire

head of the spermatozoön is present as shown by the middle piece, but it has been drawn out to a band and shows its granular structure. Fig. 19 shows a case in which the middle piece and a small part of the base of the sperm head has been entirely removed. Figs. 20, 21, 22 and 23 show the removal of increasingly large portions of the sperm head. Cases could be illustrated beyond either end of this series in which on the one hand the spermatozoön is entirely uninjured, and on the other hand even the perforatorium is pulled out of the entrance cone. The cases illustrated are merely selections from a very much larger set of observations.

It is shown, therefore, that practically any degree of injury to the spermatozoön may be produced prior to its entrance. Cases in which the delicate perforatorium is broken next the head leaving only the cone and attachment granules are the most common as is to be expected; but the other classes of injury bear witness to the tenacity of the hold of the spermatozoön on the egg.

*b. Injuries to the spermatozoön after penetration has begun.* This class of injuries is relatively rare because the actual process of penetration requires only about two minutes, and the chances of involving it are therefore correspondingly few. However, I have found a considerable number of such cases in the preparations. At first I looked to such injuries as the only source of the partial sperm nuclei already observed in the egg, but as I made the observations described in the preceding section, I realized that they were not the only or indeed the chief source of such partial sperm nuclei. Figs 24 and 25 illustrate two cases from the same experiment (6.5.1, 1911) from which figs. 18 to 23 are taken. In fig. 24 it will be seen that penetration has already begun (cf. fig. 6), and the external part of the spermatozoön is ravelled out and the middle piece, at least, lost. Fig. 25 is a little more complicated; in this case penetration had probably reached a condition intermediate between figs. 6 and 7. The centrifugal force has doubled up the part of the sperm in the egg, and drawn out the external part removing the middle piece. Fig. 26, finally, is a clear cut case, the external part alone being removed entirely, and the remainder showing a clear penetration picture. This is the prob-

able interpretation of this picture, although it was taken from a preparation killed fifteen minutes after centrifuging; further penetration following centrifuging was probably prevented in this case by the large oil drops which were driven around the entering sperm.

### 3. *Penetration of injured spermatozoa*

Injured or partial spermatozoa may enter the egg, demonstrating that penetration of the spermatozoön, after attachment is once secured, is an active function of the egg and not at all of movements of the sperm itself, if any further evidence is needed on this point. But if the entire spermatozoön be removed, the cone remains superficial and does not penetrate; at least I have repeatedly found it in a superficial position fifteen to thirty or more minutes after the normal time of penetration, and I have never found it actually penetrated unless accompanied by all or a part of the sperm nucleus.

The evidence for the entrance of injured or partial sperm heads is furnished by cytological study of eggs fixed at definite periods after centrifuging. Particularly clear evidence is furnished by one series of nine stages fixed fifteen minutes after centrifuging. In the fifth stage of this series centrifuged fifty minutes after insemination and fixed fifteen minutes later, early stages of penetration are very abundant, and among them are some that show unequivocal evidence of being partial sperm nuclei, for there are numerous cases in which an injured part of the sperm head is left outside on the membrane and thus guarantees the partial nature of the sperm nucleus within. Some such cases are illustrated in figs. 27 to 32.

Fig. 27 shows a case killed fifteen minutes after centrifuging in which the connection between the internal and external parts of the spermatozoön is entirely broken. The partial sperm nucleus within the egg and the cone have already begun to rotate. In fig. 28 we have a similar case, except that there still remains a delicate connection between internal and external parts of the spermatozoön, and this condition would lead, by rupture of this connection, to the condition shown in fig. 27. It would seem,

then, that the spermatozoön may be so injured in the process of centrifuging that it ruptures in the process of penetration, and we thus learn that partial sperm nuclei may come from spermatozoa merely injured and not actually broken in the process of centrifuging. It should also be noted that in each of these cases the internal part of the spermatozoön is divided in two parts; presumably after penetration, by constriction around an injury. Fig. 29 shows a condition similar in many respects to figs. 27 and 28, in that the entire spermatozoön is present, and a large external part is separating from a smaller internal part. Figs. 30 and 31 show the early penetration of parts of spermatozoa, recognizable as such merely by their small size in the absence of the external part. The cases illustrated in figs. 30 and 31 are such as would be derived from injuries similar to those shown in figs. 20 and 21.

Rotation of the sperm cone complex has already begun in figs. 27 to 31, while the cone is much nearer the surface of the egg than in normal fertilization. Rotation begins immediately after penetration is completed, and hence takes place nearer to the surface when portions only of the sperm are concerned than when the whole sperm is concerned. In this connection the reader should compare figs. 9 and 10.

#### *4. Stages of rotation of the partial sperm nuclei and origin of the sperm aster*

Convincing evidence of the partial nature of sperm nuclei in later stages of rotation and origin of the aster is difficult to secure, in spite of the fact that the control consisting of part of the same lot of eggs preserved immediately after centrifuging may show numerous instances of partial sperm heads like those illustrated in figs. 18 to 26. The difficulty of securing unequivocal evidence arises from the fact that mere size, except in extreme cases, is no longer a safe guide; in the first place the diameters of the nuclei vary only as the cube root of their volumes, hence considerable differences in volume may be represented by undetectable differences of diameter. In the second place the sperm nuclei are not round and all are not in favorable positions for comparable meas-

urements. In the third place the volume of the sperm nucleus normally increases considerably for some time after entrance, and the difficulty of deciding whether comparable stages are involved is sometimes great.

In spite of these difficulties, however, I have found sperm nuclei which must be interpreted as partial on the basis of their size alone, especially in later stages.

Fortunately however, it is not necessary to rely on size differences alone for in some of these stages, as in the case of the penetration stages just considered, a remnant of the sperm head may be found adhering to the membrane at the point of entrance, guaranteeing the partial nature of the sperm nucleus within. A few of these stages may be considered first:

Fig. 32 shows a case immediately continuing those described in Section 3. Here the rotation had already begun, as evidenced by the position of the cone, a very delicate connection still remaining with the external part of the sperm head impedes the rotation of the sperm nucleus within. Fig. 33 *a* and *b* shows a more advanced case; in fig. 33 *a* the cone and sperm nucleus are shown almost half rotated. One would not be able to decide from the size that it was a partial sperm nucleus, but the next section, shown in 33 *b* contains a considerable portion of the sperm head still connected with the middle piece which has remained without on the egg membrane. This is a very critical case, demonstrating that a partial sperm nucleus will rotate like a complete one. The portion in 33 *b* is entirely disconnected from the nucleus shown in 33 *a*; they are in the very act of separation. In fig. 35 we have a very fortunate section in which a completely rotated fertilization complex: cone, sperm nucleus and aster, is present, and a considerable portion of the same sperm head is found on the membrane outside the egg; the external portion is entirely comparable to the condition shown in figs. 19, 24 and 25 and there can be no question about its interpretation. Undoubtedly, the condition came from one essentially similar to that shown in fig. 25; it is, in fact, exactly what one would predict a later stage of the condition of fig. 25 to be, assuming a break to occur between the parts within and without the membrane.

Fig. 36 shows a case in which part of a sperm head has been left behind in the peripheral protoplasm at the point of entrance, and we have the completely rotated complex of cone, sperm nucleus and aster within. Another similar case is illustrated in fig. 37, and still other illustrations could be given.

It will be noted that there are very considerable size differences in the sperm nuclei of figs. 35 to 37, and all of these are distinctly smaller than the normal comparable stage shown in fig. 11. But in these cases it is not necessary to rely on the distinctly smaller size as evidence of their partial nature, for we have the evidence of sperm remnants left at the point of penetration. In figs. 38 and especially 39, however, we have cases in which the undeniably minute size of the sperm nuclei alone is sufficient evidence, taken in connection with the records of injury to the external sperm head by removal of the jelly illustrated in figs. 18 to 26, to prove that they have been derived from a mere fragment of an entire sperm head probably (in the case of fig. 39) not exceeding one-sixth to one-eighth of the bulk of the entire head, although the external part was entirely lost in this case.

It is, perhaps, not necessary, but it may be well to emphasize the fact that the cases selected for illustration are all from complete series of sections, and that the neighboring sections were always consulted for possible parts of the sperm nucleus. *The small size is not due to division by the microtome knife.* The cases described are selections from a much greater number of records.

It will be noted that if small fragments of a sperm head can produce aster formation in the egg, the possibility of polyspermy with parts of a single spermatozoön is given. This condition, which I anticipated on theoretical grounds, was finally found and is illustrated in fig. 40. There are two sperm nuclei, each with an aster, associated with a single cone. The small size of these nuclei marks them as partial, and their connection with a single cone as parts of a single spermatozoön. The only alternative explanation of this figure would be that two spermatozoa had become implanted so close together as to produce a single cone, and that they had received comparable injuries in the process of centrifuging. Against this explanation are the results of many

observations of polyspermy in none of which was implantation nearly close enough together to produce a single cone; in one series that I possess many eggs have thirty or more spermatozoa implanted, but the points of insertion are always separate. Moreover, in such a case one would expect the cone to be larger than usual, but in this case it is a little below the average size; one would also expect to find two equal groups of implantation granules with separate attachments of the nuclei, but only one group and one attachment is found here, viz., in the nucleus to the left; the granule near the right nucleus is too small to represent a separate implantation group, and moreover, it has no connection with the neighboring nucleus. Finally, it will be seen that conditions such as those illustrated in figs. 27 and 28 must inevitably lead to the condition of fig. 40 if the fragments of the sperm head do not reunite.

There is thus every reason for interpreting these two nuclei as parts of a single one. This rare find simply emphasizes the conclusions already reached concerning the fertilizing power of portions of the sperm head.

Without attempting at this place to discuss the results fully, I would, nevertheless, emphasize the two facts of greatest importance already brought out. In the first place it is shown that an apical fragment of the sperm head is able to produce an accompanying aster in the egg cytoplasm; *the sperm aster has therefore no necessary relation to the middle piece of the spermatozoon, or to the centrosome of the spermatid.* In other words, using the formation of a sperm aster as criterion, the fertilizing power of the spermatozoon is not localized in the middle piece, as supposed by Boveri and others, but is a function of even small fragments of the sperm nucleus alone. In the second place the great beauty of this material is that the orientation of the sperm nucleus, whether entire or partial, is preserved until after the origin of the sperm aster, and this enables one to determine that the sperm aster always arises in relation to the most basal point of the sperm nucleus. Altogether, I have observed well over one hundred cases of entire and partial sperm nuclei in these stages, and have never found any exception to the rule that the sperm aster arises



at the point of the sperm nucleus farthest from the cone. In other words, *the position of origin of the sperm aster is a function of polarity of the sperm nucleus*, and it is this which explains its invariable origin, so far as has been recorded in the literature, in the position of the middle piece of the spermatozoön; and the theory that a centrosome introduced by the spermatozoön is necessary for such formation is therefore shown to be incorrect.

The results so far show that sperm fragments, even of very minute size, may enter the egg in conjunction with the cone, rotate in the normal manner and produce an aster in the egg-cytoplasm. The question now arises, what is the ultimate fate of such fragments? Is their fertilizing power adequate to produce segmentation of the egg?

##### 5. *The later history of the partial sperm nuclei*

Partial sperm nuclei separate from the cone and penetrate towards the center of the egg like normal ones (figs. 38 and 39). The sperm aster divides and forms an amphiaster in the stage of the anaphase of the second maturation division characterized by inequality of the two poles as in the normal. But apparently the size of the centrosomes and the extent of the astral radiations are directly proportional to the mass of the sperm nucleus concerned. This is brought out very well in fig. 41, which is a reconstruction from three successive sections showing two sperm nuclei of unequal size in the same egg. The egg in question had been centrifuged forty-four minutes after insemination and was preserved forty-seven minutes later in the stage of the telophase of the second maturation division. It will be observed that the larger nucleus (left) is accompanied by a larger centrosome and aster than the smaller one (right), and it should be stated that the aster in each case is the larger one of an amphiaster. Here, where direct comparison between nuclei of unequal size and their accompanying asters within the same egg is possible, the proportional size of asters to nuclei is obvious. I do not mean of course to assert that the proportions are mathematically accurate for this would be impossible to determine.

In general, larger sperm nuclei are accompanied by larger asters and smaller nuclei by smaller, roughly proportional, asters, after they are fully formed. Exceptions to this rule are certainly rare. I have, however, found a very few cases, two or three in all, in which a very small sperm nucleus is accompanied by a disproportionately large aster. The explanation of such cases is uncertain, but I am inclined to attribute it to a secondary reduction of the sperm nucleus after penetration and aster formation, such as might conceivably result from some form of injury received in centrifuging.

The significance of this proportional relation is at once apparent; if the aster is a product of a nucleo-cytoplasmic reaction of some kind, as we have already seen reason to believe, there must be a quantitative relation between the product (aster) on the one hand, and the reacting elements (nucleus and cytoplasm) on the other, and this is what we find.

After maturation is completed and the germ nuclei are formed, we have to find a new criterion for partial sperm nuclei. Comparison of size of the egg and the sperm nucleus alone is not very satisfactory, because both nuclei are swelling very rapidly at this time and they may meet and begin to fuse before their enlargement is complete, so that complete identity in size of egg and sperm nucleus prior to fusion is not invariable in the normal fertilization. But a valid criterion may be found in the following phenomena: the germ nuclei are formed by chromosomal vesicles, one for each chromosome, and in each vesicle a sharply marked chromatic nucleolus arises before the separate vesicles fuse. Fusion begins very early and growth of the nucleoli accompanies it; however, an elimination or dissolution of the nucleoli begins before fusion is complete, so that their number is rapidly reduced again, and they entirely disappear before the actual prophases of the first cleavage spindle. Fusion of the two germ nuclei with one another may also begin before the fusion of the chromosomal vesicles in each is complete. Under normal conditions the number of the chromatic nucleoli is probably the same in each germ nucleus in the early stages. I therefore looked for cases of striking disparity in number between the chromatic nucleoli of

the egg and sperm nuclei. Some such cases were found, but they were much fewer in number than expected on the basis of the number of partial sperm nuclei found in earlier stages. I was therefore led to suspect that the smallest sperm nuclei might disintegrate prior to this time. However, I have failed to find direct evidence for this. It may be, therefore, that the failure to find the expected number of partial sperm nuclei in the stage in question is due to the fact that the critical period for such determination is of very brief duration.

Fig. 42 illustrates a case of disparity between the two germ nuclei. Five sections are involved, and the male and female nuclei are indicated by the appropriate signs. The male component, distinguished by its accompanying aster, exhibits five nucleoli and the female thirteen. The volume of the female component is also much greater than that of the male component. I believe, therefore, that we have here an undeniable case of fusion of a partial sperm nucleus with an entire egg nucleus.

In the stage of the first cleavage spindle of eggs centrifuged just before penetration of the spermatozoön we have three classes of eggs, aside from a very few polyspermic eggs: (1) Some with a more or less normal cleavage spindle; (2) some with a monaster centered in a group of chromosomes; (3) some without any trace of astral radiations although chromosomes are formed. In a particular lot of eggs (7.4 of 1911) of which 10 per cent segmented, at the stage of the first cleavage the first class is rare, the second class is quite common, and the third is the most frequent condition. The first class evidently corresponds to the 10 per cent of eggs that segmented.

The two latter classes are illustrated in figs. 43 and 44. Fig. 43 is a camera drawing of the three sections of the nucleus of a single egg belonging to the third class. The eggs of the first class of the same lot were in various stages of the anaphase and telophase of the first cleavage. There appear to be fourteen chromosomes, the number usually found in the maturation spindles; the position near the animal pole proves it to be the egg nucleus. The absence of the sperm nucleus is readily demonstrated. There is an entire absence of all radiations in the cytoplasm. Both

polar bodies are present. This condition, as I have already said, is the commonest condition in such a lot of eggs, and both polar bodies are invariably present.

Attention may be directed to the fact that each chromosome is embedded in a homogeneous ground substance of about the same tint, in the iron haematoxylin stain employed, as the cytoplasm. Evidently, each chromosome with its surrounding matrix corresponds to a single chromosomal vesicle of the early egg nucleus; the numbers are the same. The chromosome of the succeeding cell generation arises within the substance of the chromosome of the preceding generation in this case.

The second class of cases, monasters, is illustrated in fig. 44. I was at first inclined to think that these might be due to fertilization with partial sperm nuclei, especially as the degree of development of the monaster shows a wide range of variation. But careful study of the eggs concerned showed that the first polar body had invariably failed to form, and the second was always present alone. About twenty-five cases of this kind have been examined without a single exception occurring.

In the second study of this series (Lillie, '11) I have described the cause of failure of the first polar body. This condition occurs in eggs centrifuged just before the formation of the first polar body. The first maturation division may then take place within the egg forming two nuclei, and the second maturation spindle which involves both nuclei is a tetraster (Lillie, '11, fig. 6). The second polar body is formed from one pole of the tetraster and three nuclei are left in the egg, which soon unite. Under these circumstances, if the sperm nucleus be absent, a more or less feeble monaster may develop at the time of the first cleavage; though in a few cases where only the second polar body was formed, no signs of radiations were found.

There is thus a more or less striking difference in the behavior of the egg nucleus in those cases where both polar bodies are formed and those in which the first polar body is suppressed. It is a very interesting problem whether the formation of the monaster in the latter case is a purely quantitative relation, due to the larger number of chromosomes present in such cases? There

is considerable variation in such cases in the size of the second polar body and the quantity of the chromatin which it contains, and corresponding differences in the number of chromosomes left in the egg. But the degree of development of the monaster is not a function of the number of chromosomes in the egg; and there are cases in which no aster formation is associated with a larger number of chromosomes in such eggs, and a well developed monaster with a smaller number. It is possible that there may be a qualitative relation depending on what chromosomes are extruded in the second polar body; but in view of the complicating considerations resulting from possible injurious effects of centrifuging itself, no definite conclusion on this point seems possible.

It is, in any event, certain that the sperm nucleus is absent in both the second and third classes of eggs.

As regards the effect of the partial sperm nuclei on the cleavage process we are therefore reduced to the class of cases in which a cleavage spindle is actually formed. In observing the living eggs I was struck with the fact that the cleavage of many centrifuged eggs tends to be irregular or partial, especially of those centrifuged at the time when injuries to the spermatozoon were to be expected. And in the sections I find many cases of partial cleavage. The cleavage of many also stops in the two-cell stage. It is natural to suppose that such partial cleavages are the result of fertilization with partial sperm nuclei, seeing that we know from the data recorded above that there is not even the least indication of cytoplasmic cleavage in the entire absence of the sperm nucleus. A rigorous demonstration of such a conclusion would, however, require a cytological analysis in which the number of chromosomes in the different cleavage spindles of normal and partially segmenting eggs should be compared; the relative sizes of the karyokinetic figure, and possibly other data, should also be taken into account. Unfortunately, the material preserved for this study is in too advanced a stage to make an exhaustive study of these relations, and this part of the investigation must therefore be postponed.

It must be admitted that other causes than fertilization with partial spermatozoa might be responsible for the partial cleavage.

e. g., injury of certain kinds caused by the centrifuging, possibly abnormal maturation, or a general systemic disturbance of the cytoplasm. But this does not seem very probable, since eggs centrifuged at times when injury to the spermatozoön is not to be anticipated do not exhibit the partial cleavage, at least to the same extent. I would therefore regard it as probable, though not proved, that partial sperm nuclei tend to produce more or less defective cleavage.

#### GENERAL DISCUSSION

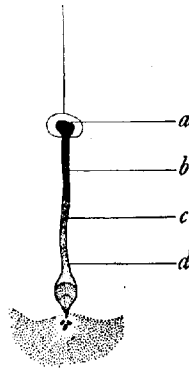
##### 1. *The centrosome theory of fertilization*

The centrosome theory of fertilization is still accepted by most morphologists in spite of the doubts that have been thrown on the theory of the permanence and genetic continuity of the centrosome by the production of asters in unfertilized eggs (Morgan, Wilson, etc.), and by the studies in artificial parthenogenesis. The theory that the spermatozoön introduces an extra-nuclear centrosome destined to become the organ of cell division of the oöperm is confronted for the first time, in the results of the foregoing study, with a crucial experimental test. And the result that even small parts of the sperm head produce a typical centrosome and aster in the egg cytoplasm conclusively demonstrates the inadequacy of this conception of fertilization. So long as similar experiments on other forms are lacking, there is no reason to believe that the production of the sperm centrosome depends upon any different principle in *Nereis* than in other forms.

Meves ('11a) demonstrates that the sperm aster does not arise in connection with the so-called middle piece of the spermatozoön in the case of the sea-urchin, but at the base of the sperm nucleus itself, the middle piece being already separated from the nucleus and lying to one side at the time that the sperm aster arises. These results, while inconclusive in themselves, are nevertheless distinctly unfavorable to the existence of an extra-nuclear centrosome as the cause of formation of a sperm aster in this classical case. Further, one can say without fear of successful contradiction that in no animal has it been shown that the

sperm aster arises around an extranuclear centrosome of spermiatic origin. There is, therefore, no reason for assuming that the experiments on *Nereis* have revealed an exceptional condition.

Either, then, the centrosome theory of fertilization must be rejected in toto, or the sperm nucleus must be regarded as a centronucleus in Boveri's sense (Boveri '00), i. e., as including the centrosome. If we examine the latter point of view we see that it would be necessary to assume that centrosomes exist in the sperm nucleus at every level, for the sperm aster can form at any level as demonstrated by the experiments. The diagram (text fig.



Text fig. 4

4) will make this clear. It represents the sperm nucleus in the form that it possesses as penetration is nearly completed (cf. fig. 8); normally the sperm aster arises at point *a*, but if *ab* be removed then at point *b*, if *ac* be removed at *c*, if *ad* be removed at *d*, etc. But the assumption that centrosomes exist throughout the nucleus and condition this phenomena would seem to be merely an exaggerated morphological point of view with reference to a problem that is, after all, fundamentally physiological, viz.: by virtue of what properties does the sperm nucleus exercise this effect? If the great mass of experimental cytological data favored the view of the permanence of the centrosome, the con-

ception of the centronucleus might legitimately be extended to cover the present case, but this cannot be said to be true; and in the present state of our knowledge such an explanation would appear forced and merely formal.

The formation of the sperm aster takes place on the boundary between nucleus and cytoplasm, and as we have seen (fig. 41), there are very definite quantitative relations between the bulk of the partial sperm nuclei and the degree of development of the aster and the size of the centrosome. This relation leads to the conclusion that if intranuclear centrosomes are the causes of the formation of the sperm aster, not only must they exist at every level, but also that they must decrease in size from the base to the apex of the sperm nucleus! The formation of the sperm aster on the boundary between nucleus and cytoplasm and the quantitative relations existing between size of the nucleus and of the aster demonstrate, it seems to me, that the centrosome and aster owe their existence to an interaction between nucleus and cytoplasm, and not to any third element. All the observed relations in the case of *Nereis* harmonize with this point of view.

The production of astrospheres remote from the nucleus in the experiments of Morgan ('96 and '99) and others show, it is true, that the nucleus is not necessary for the production of such phenomena. These asters are apparently very temporary formations, and evidence that their central bodies may divide like the centrosome of the sperm aster, or other centrosomes associated with nuclei, is lacking. Nevertheless, it seems probable that fundamentally similar physiological causes are at the foundation of both sets of phenomena, and we can only assert our profound ignorance of what these causes really are.

## *2. The polarity of the sperm-nucleus*

We may use the term polarity to describe the fact that the sperm aster arises invariably at the most basal point of the sperm nucleus, whether it be entire or partial. This phenomenon corresponds accurately to the general features of polarity of ova or lower organisms, as, for instance, the formation of oral organs at the oral end of a cut piece, etc.



It might, perhaps, seem possible at first thought that the aster appears in this position with reference to the partial sperm nuclei because we have here a broken surface; this is at least a condition in which this particular point of the surface of the partial nuclei differs from the remainder of its surface. But in the entire sperm nucleus, where there is no broken surface, the position is always the same. Moreover, some ten or fifteen minutes elapses after entrance of the sperm head before the aster becomes visible, and in this time the nucleus has changed form so as to produce a pointed extremity (figs. 33 *a*, 36, 37, 40, etc.) in the position where the aster is to appear whether the nucleus be entire or partial. This is perhaps sufficient evidence of repair of the wounded surface.

We have to seek some more profound cause for this localization, and I believe that it must be regarded as a special case of organic polarity to be explained like other cases on the basis either of gradation or orientation of materials. From this point of view the nucleus would possess an immanent structure determining the location of aster formation and therefore the plane of division of the nucleus.

### *3. Theory of fertilization*

I have pointed out repeatedly in these studies that fertilization involves two phases, viz.: an external phase prior to entrance of the spermatozoön, in which certain cortical changes are produced in the egg, and an internal phase, following penetration, involving a complex series of phenomena. As I pointed out in the introduction, this paper is a contribution to the analysis of the second phase. On the basis of experiments on artificial parthenogenesis and hybrid fertilization, Loeb has made a similar distinction of two phases, and so far the results of what we may call the biological and the physico-chemical analyses of fertilization are in accord.

If we reject the centrosome theory of fertilization, as I believe we are compelled to do, what point of view from the side of the biological analysis shall we put in its place? The theory of the

internal phase of fertilization must proceed from the fundamental fact of the difference in behavior of the sperm nucleus and the egg nucleus in the cytoplasm of the egg. The former induces aster formation and karyokinesis; the latter does not. Let us recall the facts in the case of *Nereis* briefly again: (1) Even minute fragments of the sperm nucleus cause the formation of an aster with a centrosome capable of division. (2) If the sperm nucleus be prevented from entering, the egg nucleus may indeed form chromosomes but no aster arises, provided that both polar bodies are formed.<sup>2</sup> (3) We may add from experiments on other forms that in the absence of the egg nucleus the sperm nucleus behaves the same as in its presence.

Clearly, then, there is some difference, associated with their sex-origin, between these two nuclei; and the most direct form of interpretation of this difference is that which identifies it with the fundamental sex characters which inhere in every cell. In other words, the sperm nucleus has the character maleness, whatever that may be, and neither the egg nucleus nor cytoplasm possesses this character. It makes no difference that half the spermatozoa may carry the *factor* for femaleness and half for maleness. The distinction between *character* and *factor* is clear. It may be, on the other hand, that ova and spermatozoa acquire in the course of gametogenesis special differentiating properties that are the cause of the fertilizing power of the spermatozoön.

However we may conceive the demonstrated difference between the sperm nucleus and the egg nucleus, it is obvious that there is a lack of interchange between the egg nucleus and the egg cytoplasm that conditions the inhibition of the unfertilized egg. In some way, then, the maturation divisions of the egg must have removed certain reacting constituents of the germinal vesicle, or have brought about certain cytoplasmic changes in the egg, because we have perfect karyokinetic phenomena in the maturation divisions and a sudden cessation thereafter.

<sup>2</sup> The monaster that arises after suppression of the first polar body with prevention of entrance of the sperm nucleus forms a special problem which we need not consider here.

But it is clear at least that the maturation of the egg does not differ from the maturation of the spermatozoön in this respect. In both cases capacity for further cell-division is lost after the second maturation division, and it is quite natural, certainly, to postulate similar causes for this phenomenon in both sexes. If, as the results of the present study indicate, karyokinesis is the result of a certain *qualitative* nucleo-plasmic relation (to be distinguished from R. Hertwig's *quantitative* nucleo-plasmic relation) we have to postulate in both cases a disturbance of this relation. And as this relation must be conceived as a chemical interaction of some kind, precipitated possibly by rhythmical changes of permeability of the nuclear membrane, the alteration in question must involve either the establishment of an impermeable condition of the nuclear membrane or a chemical change in nucleus or cytoplasm. But we have seen in *Nereis*, that, even when the membrane of the nucleus of the mature egg breaks down, no karyokinetic phenomena follow, unless the egg is fertilized. So the phenomenon of cessation of division can hardly be conceived as conditioned by the membrane alone.

The egg-cell and the spermatid are not the only cells that lose the capacity for division in the course of development. In the course of senescence all cells lose this capacity, and studies in cell-lineage have shown that certain cells entirely lose the capacity for division in very early stages. I need cite only the case of the so-called turret cells in *Crepidula* (Conklin '97), which are formed in the sixteen cell stage, and which divide only twice during the cleavage period. Mead has called attention to similar cases in his studies of cell-lineage in Annelids (Mead '98). Cessation of division cannot be a problem of centrosome or no centrosome in such cases; nor yet in the case of the spermatid. A much more profound physiological cause must be involved.

Constructive metabolism has come essentially to a standstill in the mature gametes; the rate of metabolism in the mature unfertilized egg as tested by oxygen consumption is many times less than that of the fertilized egg (Warburg, '05). Child ('11) cites as conditions that lower the rate of metabolism, decrease in permeability, increase in density, accumulation of relatively inactive

substances, etc., but we know that constructive metabolism is also impossible in the absence of a nucleus, and we may conclude from many facts, as Conklin ('12) expresses it, that "rapid and intimate interchange between the chromatin and the protoplasm is the condition of rapid metabolism and ex hypothesi of rejuvenescence; slow interchange is the condition of slow metabolism, and of senescence." It is on account of the slowness of such interchange between nucleus and cytoplasm, as I believe, that the unfertilized egg is inhibited from development. The internal function of the spermatozoön in development is to restore the condition of active and intimate interchange between nucleus and cytoplasm. Aster formation and karyokinesis are evidences of such restoration. The sperm nucleus and egg cytoplasm are immediately capable on union of such interchange, and as the fertilization process proceeds the egg nucleus is drawn in.

We are led, then, to the following point of view with reference to the internal phenomena of fertilization, viz.: in both the sperm and the egg cell as the result of maturation the capacity for the nucleo-plasmic interaction necessary for construction metabolism has been lost. But such interaction takes place between the sperm nucleus and egg cytoplasm, and this initiates the internal phenomena of fertilization. The egg nucleus also is drawn into the karyokinetic phenomena in one of two ways, either that the sperm nucleus has so altered the egg-cytoplasm that karyokinetic reaction between the egg-nucleus and its own cytoplasm can now follow, or that copulation of the germ nuclei results in a change in the egg nucleus that restores its capacity for the necessary nucleo-plasmic reaction.

In his experiments on constricting fertilized eggs of the sea-urchin between the germ nuclei, so that the copulation of the latter was prevented, Ziegler ('98) has shown that the egg nucleus becomes surrounded by cytoplasmic radiations which rhythmically appear and disappear synchronously with disappearance and reappearance of the nuclear membrane. These observations indicate a change produced by the sperm nucleus throughout the egg cytoplasm, inducing partially but not completely the rhythmical series of successive karyokinetic divisions. Other obser-

vations too numerous to mention demonstrate a very profound effect of the sperm nucleus on the egg cytoplasm, perhaps none more strikingly than Herlant's ('11) recent observations on the control of definite cytoplasmic areas (spermatic energids) by spermatozoa in di- and tri-spermic eggs of the frog.

Ziegler's observations then indicate that the egg nucleus reacts to the egg cytoplasm when altered by the spermatozoön, but *incompletely*. It seems probable, therefore, that copulation of the germ nuclei also involves an interaction between them that completes the fertilization phenomena. It is interesting to note that, though the chromosomes form in *Nereis* from the egg nucleus after the spermatozoön has been removed, they are not set free in the cytoplasm as they are after copulation with the sperm nucleus, but each is embedded in a matrix, and thus presents quite a different appearance from the normal. We may perhaps find in this fact, indicating lack of reaction between the chromatin and the cytoplasm, some evidence that the completion of fertilization involves interaction between the germ nuclei also.

It remains to inquire briefly how this analysis compares with the analysis of fertilization given by experiments in artificial parthenogenesis? In the first place we may note again that there is perfect agreement in the general fundamental distinction of two phases in the fertilization process as made first by Loeb, viz.: the cortical change which may be induced before penetration, and the internal changes, which follow penetration. As regards the cortical change, the view of Loeb ('09) that it is essentially a cytolytic change appears to me less fundamental than the view of R. S. Lillie ('11), that it is essentially an increase of permeability. One can readily understand that cytolysis should follow very rapidly on an increase of permeability induced by chemical means, which may be much greater than that normally induced by the spermatozoön, if such increase be not secondarily regulated. And in any event, if interchange between the egg and its medium be set up by increase of permeability, in a condition of inactivity of the nucleus, such as exists in the unfertilized egg, the resulting metabolism must be of a destructive character and so lead to a relatively rapid death of the egg as compared with eggs in which

the cortical changes have not been induced. The conception of Bataillon ('10), moreover; that the egg excretes certain inhibiting substances contained in its cortex, as a result of the cortical change, is quite readily included in this point of view; indeed, the jelly excreted by the egg of *Nereis* as a result of the external stimulus of the spermatozoön would obviously hinder free interchange between the egg and the medium so long as it exists within the egg as a thick cortical layer.

The second phase in fertilization has been treated by Loeb and R. S. Lillie. Loeb's interpretation is that the second agent in artificial parthenogenesis serves to check the tendency to cytolysis set up by the first agent; and he extends this point of view to the two phases of normal fertilization. In this opinion he is followed by Godlewski ('11), who has shown that the cytolysis, which follows on fertilization of sea-urchin eggs with sperm of *Chaetopterus*, can be checked, and parthenogenetic development induced, by a brief treatment of such cross fertilized eggs with hypertonic sea-water. R. S. Lillie ('11) regards "the critical change in the egg, to which membrane formation and the initiation of cleavage are due, as a well marked and rapid increase in the permeability of the plasma-membrane." This tends "to destroy the normal osmotic equilibrium and allow abnormal diffusion of substances into and out of cells" leading to derangement and eventual destruction of the chemical organization of the latter. And he regards it as an unavoidable conclusion that one essential effect of the after treatment with hypertonic sea-water is to restore the normal permeability.

There is considerable similarity in these points of view; both regard the second agent in parthenogenesis essentially as a regulatory factor. Godlewski's very striking results on the combination of cross fertilization and artificial parthenogenesis lead to the same kind of conclusion; to quote this author ('11):

Wir haben gesehen, dass weder die Kreuzbefruchtung allein, noch die so kurz dauernde Exposition in hypertonischer Lösung allein ausreicht, um die Entwicklung auszulösen. Erst durch die Kombination der beiden Faktoren ist der ausreichende Anstoss zur Entwicklung gegeben. Ich sehe in diesen Tatsachen die Bestätigung der von J. Loeb aufgestellten Hypothese, dass der Process der Entwicklungserregung sich in zwei

Momente zerlegen lässt—im ersten findet die Cytolyse, im zweiten die Regulierung der im ersten Akt bereits eingetretenen inneren Reaktionen im Eiorganismus statt.

There is nothing in the results of these authors inconsistent with the idea that the regulating effect of the second agent in artificial parthenogenesis may be attained through the re-establishment of normal interchange between the nucleus and the cytoplasm, which is the view to which I have been led by the results described in this series of papers. And this suggestion contains the possibility of a complete accord between the results of the analysis of fertilization by methods of artificial parthenogenesis, and the more direct method of analysis contained in my papers.

If the egg be put in a healthy metabolic state by the resumption of normal nucleo-plasmic relations following the penetration of the spermatozoön, it can no doubt regulate its own external affairs. And so it seems to me that the regulation of cortical permeability and cytolysis is probably a secondary effect of the re-establishment of normal nucleo-plasmic interchange. Even in artificial parthenogenesis, where the sperm nucleus is lacking, the action of the second agent may proceed in the same way, by causing the re-establishment of normal interchange between the egg nucleus and cytoplasm, restoring thus healthy conditions, which then regulate the cortical changes. But whether the tendency to cytolysis be checked thus secondarily, or by some direct effect of the second agent on the cytoplasm, development certainly cannot proceed without the establishment of normal metabolic interchange between the nucleus and the cytoplasm, and this must certainly be regarded as a fundamental function of the second agent in artificial parthenogenesis.

To sum up the conclusions in a sentence we may say: the action of the spermatozoön in fertilization involves two distinct phases, the first of which may be effected before penetration and brings about a sudden and marked increase in permeability of the egg-membrane; the second, which follows after penetration, consists essentially in the establishment of normal interchange between nucleus and cytoplasm, and consequent normal regulation of all the activities of the cell.

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#### DESCRIPTION OF PLATES

All figures were drawn with the camera at stage level with Zeiss Apochromat 1.5 mm. oil immersion objective, and No. 12 compensating ocular, except where otherwise stated. All figures, except 1, from sections of inseminated eggs of *Nereis limbata*. All sections from eggs killed in Meves' fluid and stained in iron haematoxylin. Plates 1 to 5 illustrate the third study; 6 to 11 the fourth study.

## PLATE 1

### EXPLANATION OF FIGURES

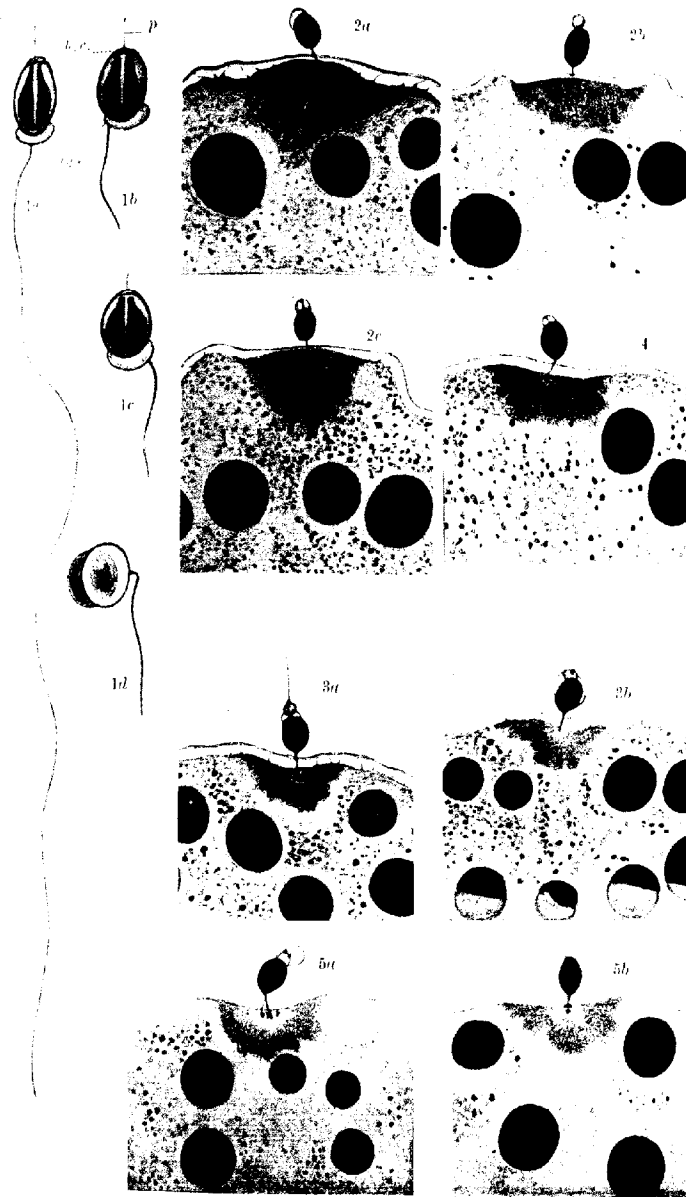
1 Spermatozoa of *Nereis* from preparations fixed in Gilson's fluid and stained in safranin and Sure-violet. 1 *a*, the entire spermatozon; 1 *b* and 1 *c* show the excentric attachment of the tail to the middle piece; 1 *d*, basal view of the sperm-head showing ring-shaped middle piece and attachment of tail. *h. c.*, head-cap; *m. p.*, middle-piece; *p.*, perforatorium. It may be noted here that the form of the perforatorium in the living spermatozon is more like the spike of a helmet.

2 *a*, 2 *b*, 2 *c* From three eggs fifteen minutes after insemination. The entrance cone is well developed and stains dark, homogeneous. The perforatorium has pierced the membrane, at least in 2 *a* and 2 *c*, but is not embedded in the cone. Note variations in appearance of the middle-piece. Tail not seen.

3 *a*, 3 *b* From two eggs twenty-seven minutes after insemination. The cone is retracted, but stains as before. The perforatorium stains more strongly than in fig. 2, but it has not yet entered the substance of the cone. The tails were very distinctly seen in these preparations.

4 Thirty-four minutes after insemination. The perforatorium has now entered the substance of the cone, and granules are beginning to appear in a smaller lighter area of the cone surrounding its tip.

5 *a*, 5 *b* From two eggs thirty-seven minutes after insemination. The clear space in the cone is now larger and the granules at the tip of the perforatorium more numerous; 5 *a*, from an almost tangential section, so that the vitelline membrane was obscured. Note the double perforatorium; 5 *b*, middle piece shows no granules; cf. 2 *b*.



## PLATE 2

### EXPLANATION OF FIGURES

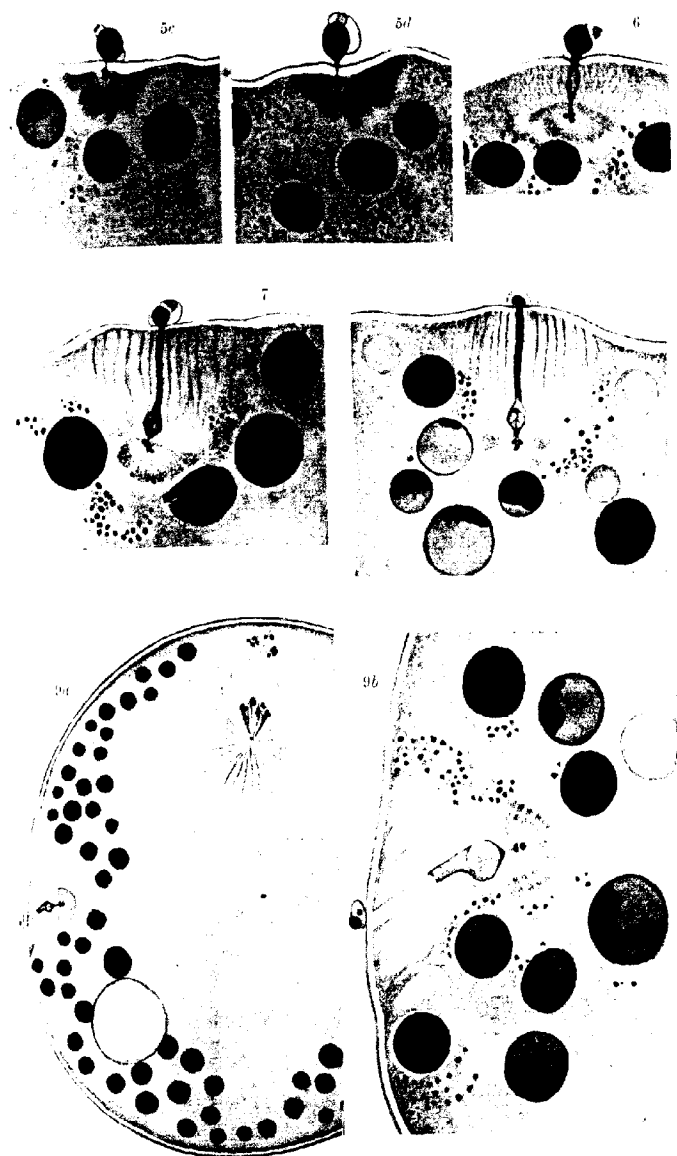
5 *c* and 5 *d* From two eggs thirty-seven minutes after insemination; cf. 5 *a* and 5 *b*.

6 Forty-eight and one-half minutes after insemination. The entrance cone has begun to sink into the egg, drawing the head of the spermatozoön after it.

7 Forty-eight and one-half minutes after insemination. Later stage of penetration of the spermatozoön.

8 Forty-eight and one-half minutes after insemination. Still later stage of penetration.

9 *a* and 9 *b*. Two drawings of the same section; 9 *a* drawn with Zeiss 2 mm. apochromatic objective and no. 4 compensating ocular; 9 *b*, the part of 9 *a* containing the spermatozoön, drawn with Zeiss 1.5 mm. apochromatic objective and no. 12 compensating ocular. Fifty-four minutes after insemination. The sperm head has completed its penetration, and its base is some distance from the periphery; but the middle-piece remains outside. Anaphase of the first maturation division.



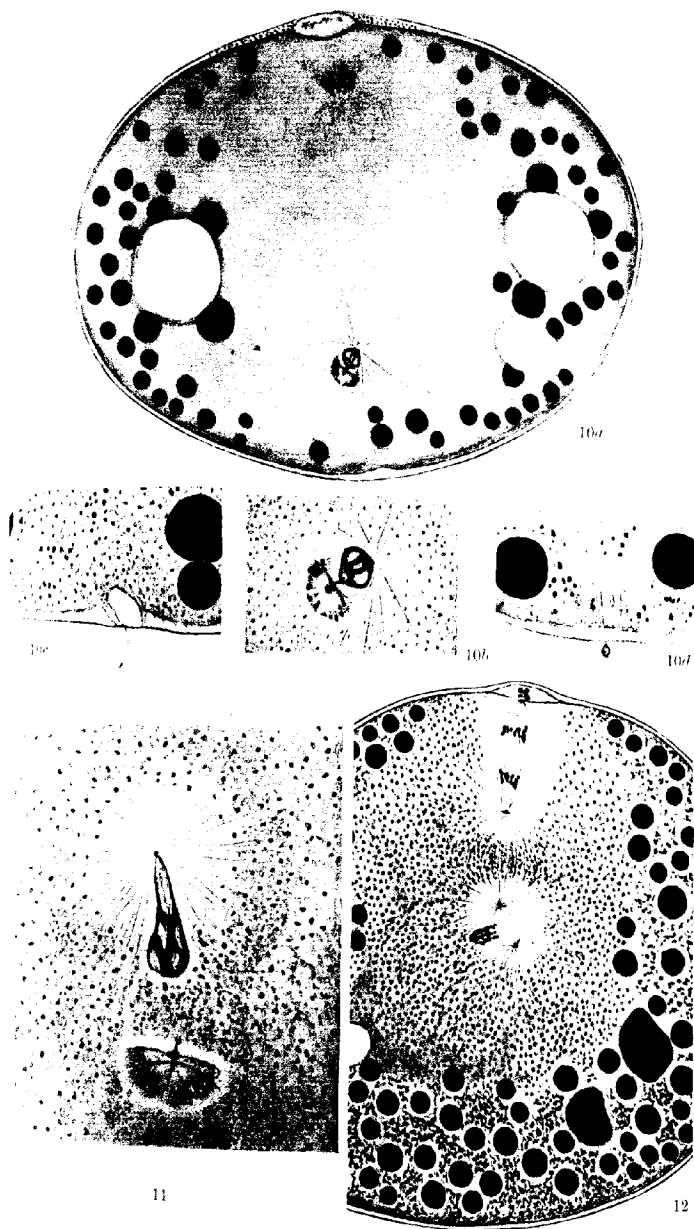
### PLATE 3

#### EXPLANATION OF FIGURES

10 *a*, 10 *b*, 10 *c*, 10 *d* Four drawings from the same section; 10 *a* drawn with Zeiss 2 mm. apochromatic objective and No. 6 compensating ocular; 10 *b*, 10 *c*, 10 *d* drawn with Zeiss 2 mm. apochromat and no. 12 compensating ocular; 10 *b* is the sperm nucleus of the same egg; 10 *c* and 10 *d* are from the following sections to show the entrance point with tail and middle piece on the membrane. Fifty-four minutes after insemination. Rotation of the sperm head and cone, and origin of the sperm aster from the pole of the nucleus opposite to the cone. Prophase of the second maturation division.

11 Sixty-four minutes after insemination. The sperm head has penetrated within the layer of yolk, and has separated from the cone, which does not appear after this stage. In the next section another sperm centrosome and aster appear; the sperm centrosome has divided.

12 Sixty-seven minutes after insemination. Sperm amphiaster with equal poles; spindle formation beginning between egg and sperm centrosomes. Anaphase of second maturation spindle. Drawn at stage level with Zeiss 2 mm. apochromatic objective and compensating ocular no. 6.



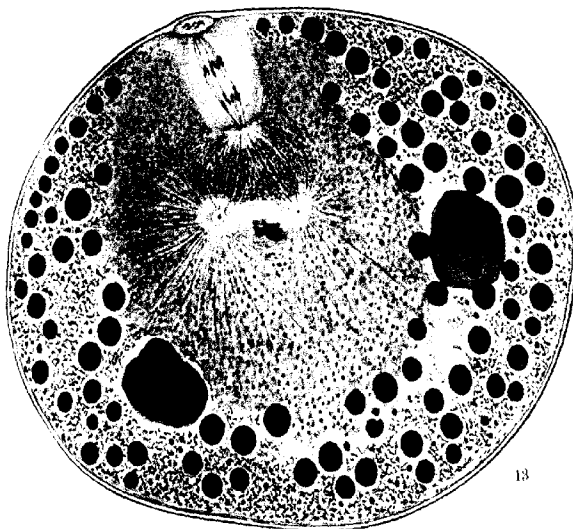


## PLATE 4

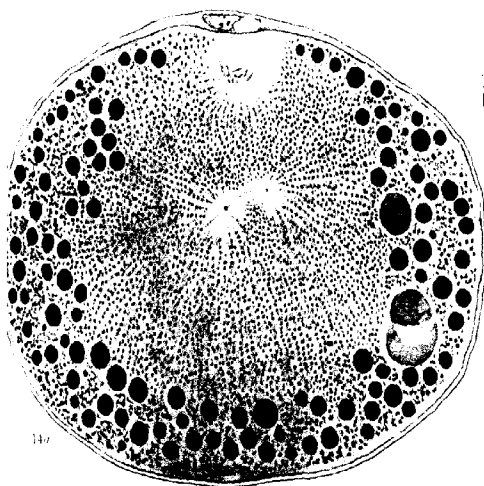
### EXPLANATION OF FIGURES

13 Sixty-seven minutes after insemination. Later stage of sperm amphister with unequal centrosomes and asters. Double spindle formation between egg center and sperm centers. Drawn at stage level with Zeiss 2 mm. apochromat objective and compensating ocular no. 6.

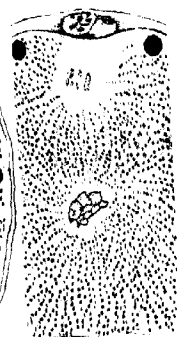
14 *a* and 14 *b* Seventy-seven minutes after insemination. Two successive sections of the same egg. The second polar body is just formed. The egg aster is beginning to degenerate. The sperm asters are also less developed than previously, especially in the case of the smaller one. Sperm amphister in 14 *a*; sperm nucleus in 14 *b*. Drawn at stage level with Zeiss 2 mm. apochromat objective and compensating ocular no. 6.



13



14a



14b

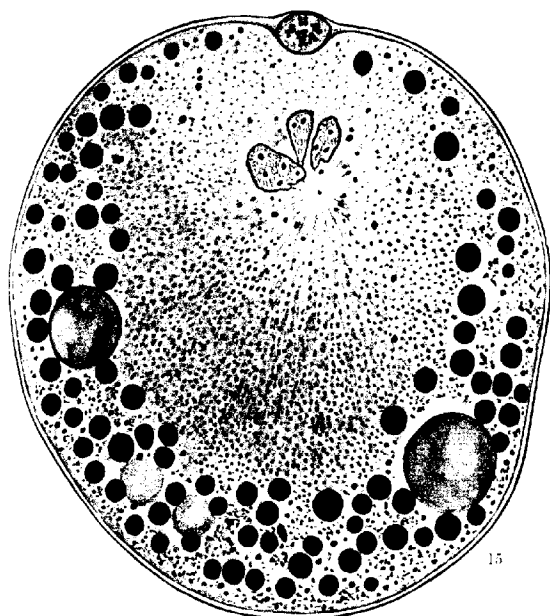
## PLATE 5

### EXPLANATION OF FIGURES

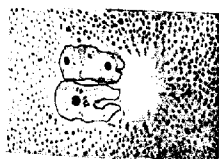
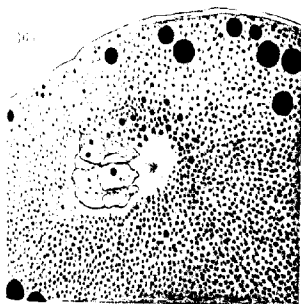
15 Seventy-seven minutes after insemination. The two nuclei to the right above are parts of the egg nucleus not yet fused together. The sperm nucleus to the left below. The aster is the larger sperm aster; the smaller one could not be found. Drawn at stage level with Zeiss 2 mm. apochromat objective and compensating ocular no. 6.

16 *a* and 16 *b* Two successive sections of the germ nuclei, seventy-seven minutes after insemination. There is but a single aster derived from the larger sperm aster. Note the black granules in the neighborhood of the germ nuclei and aster. Drawn at stage level with Zeiss 2 mm. apochromat objective and compensating ocular no. 6.

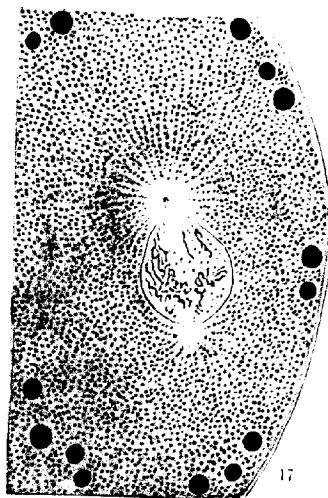
17 Origin of the cleavage centers. The partition between the germ nuclei has disappeared. The larger aster is derived from the larger sperm aster. Drawn at stage level with Zeiss 2 mm. apochromat objective and compensating ocular no. 6.



15



16b



17

## PLATE 6

### EXPLANATION OF FIGURES

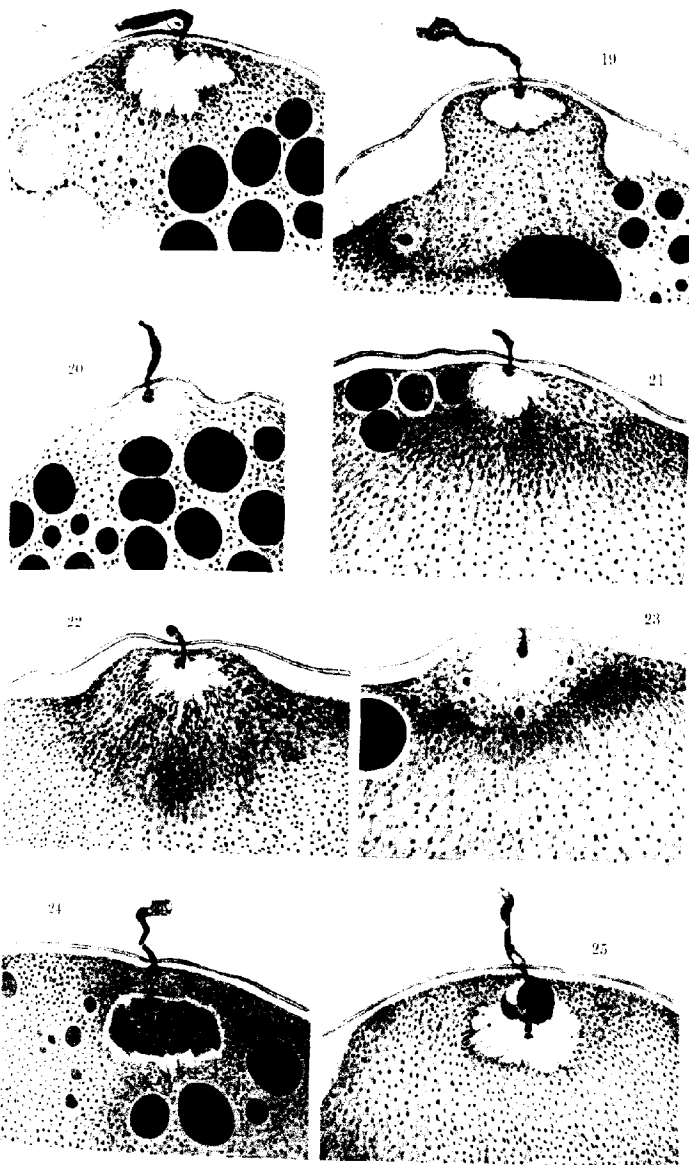
18 to 25 Show the effects of removal of the jelly by centrifuging on the spermatozoon attached to the egg. It will be noticed that the cone in this and the following plates, though just as well defined as the preceding, stains differently. The behavior of the cone is the same, however.

18 Entire spermatozoon present, drawn out to band. History: centrifuged 7200 revolution in thirty-five seconds, fifty minutes after insemination; preserved immediately.

19 The middle-piece and part of the base of the spermatozoon have been removed by the jelly. The protoplasm surrounding the cone has been raised in a protuberance, which happens not infrequently (cf. fig. 22). History same as fig. 18.

20 to 23 These figures show removal of increasingly large portions of the sperm head by the jelly. Each drawing from a single section of a separate egg. History same as fig. 18.

24 to 25 To show effects of removal of jelly by centrifuging after penetration has begun. History same as fig. 18.



## PLATE 7

### EXPLANATION OF FIGURES

26 Removal of external part of the spermatozoön by centrifuging in an advanced stage of penetration (cf. fig. 7). History: centrifuged 7200 revolutions in forty seconds, fifty minutes after insemination. Preserved fifteen minutes later.

27 to 31 To show early penetration of injured or partial spermatozoa. History: centrifuged 7200 revolutions in forty seconds, fifty minutes after insemination. Preserved fifteen minutes later.

27 Part of the spermatozoön has entered. The remainder is shown external to the membrane. The internal part is definitely divided in two. Rotation is beginning.

28 The part of the spermatozoön external to the membrane is nearly separated from the internal part, which is itself definitely divided in two. All parts a little swollen as shown by the tone of the stain. The rotation of the cone is beginning.

29 The internal part is apparently breaking off from the much larger external part of the spermatozoön. Cone in process of rotation.

30 A case in which only a small part of the spermatozoön has entered; the rest of the spermatozoön is lost. It represents a later stage of an injury similar to that shown in figs. 20 or 21.

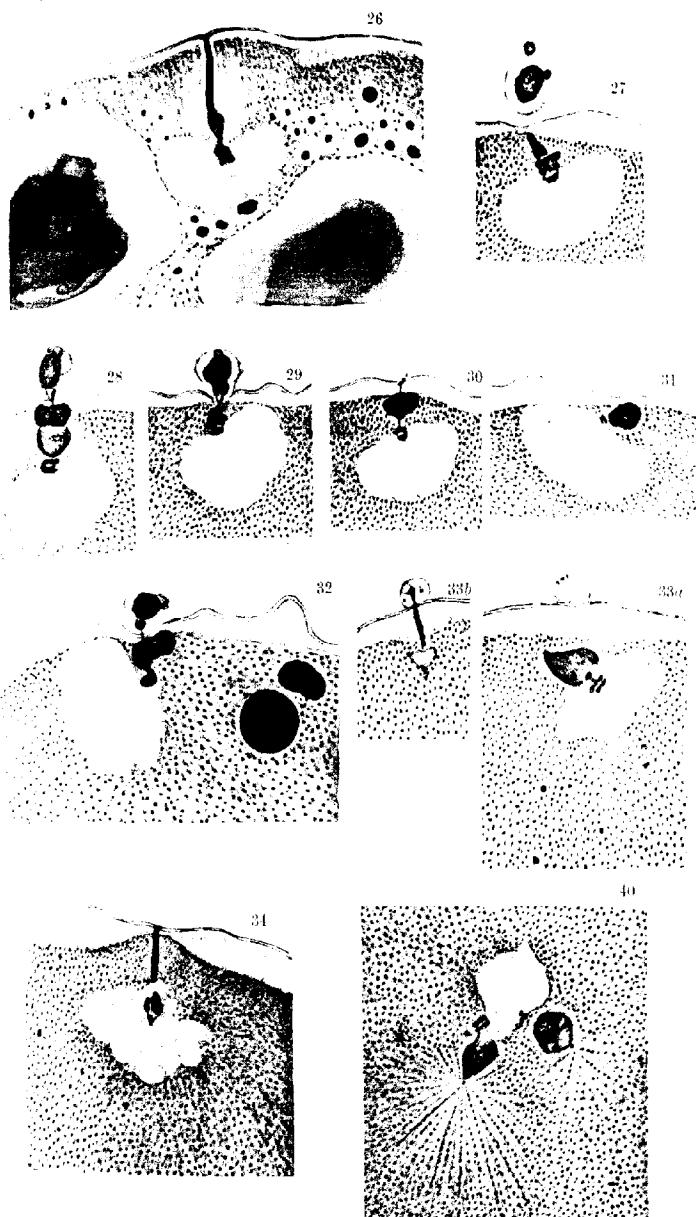
31 A case similar to fig. 30.

32 A somewhat later state of rotation of the cone than shown in preceding figures. History the same.

33 *a* and 33*b* Two successive sections of the same egg; the parts of the spermatozoön shown in the two sections are entirely separate. The proximal larger part (33 *a*) is proceeding with its rotation and development, leaving the base of the sperm head and the middle piece behind. History same as figs. 27-31.

34 Penetration stage of a sperm remnant preserved fifteen minutes after centrifuging.

40 Two partial sperm nuclei with asters associated with a single cone. Probably a later stage of a condition like that shown in figs. 27 or 28.

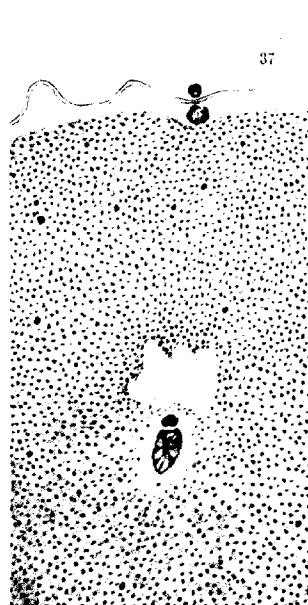
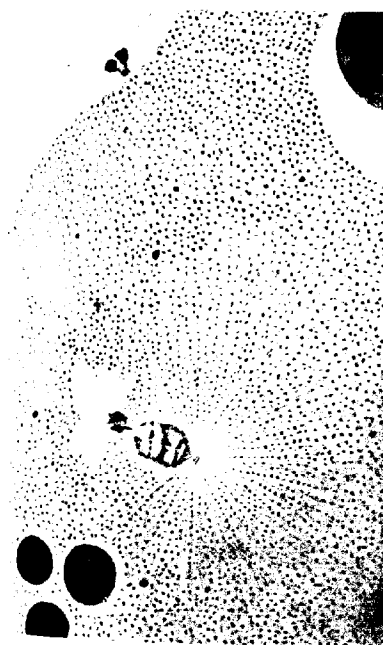
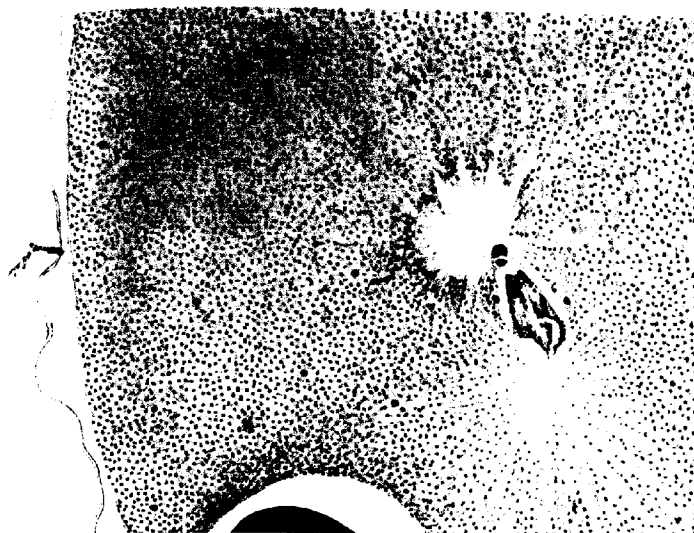




## PLATE 8

### EXPLANATION OF FIGURES

35 to 37 To show origin of asters in connection with partial sperm nuclei. In each case a remnant of the spermatozoön on the surface guarantees the partial nature of these sperm nuclei; note the variation in size. History: Centrifuged 7200 revolutions in forty seconds, fifty minutes after insemination; preserved fifteen minutes later. Fig. 36 is a combination of three sections.

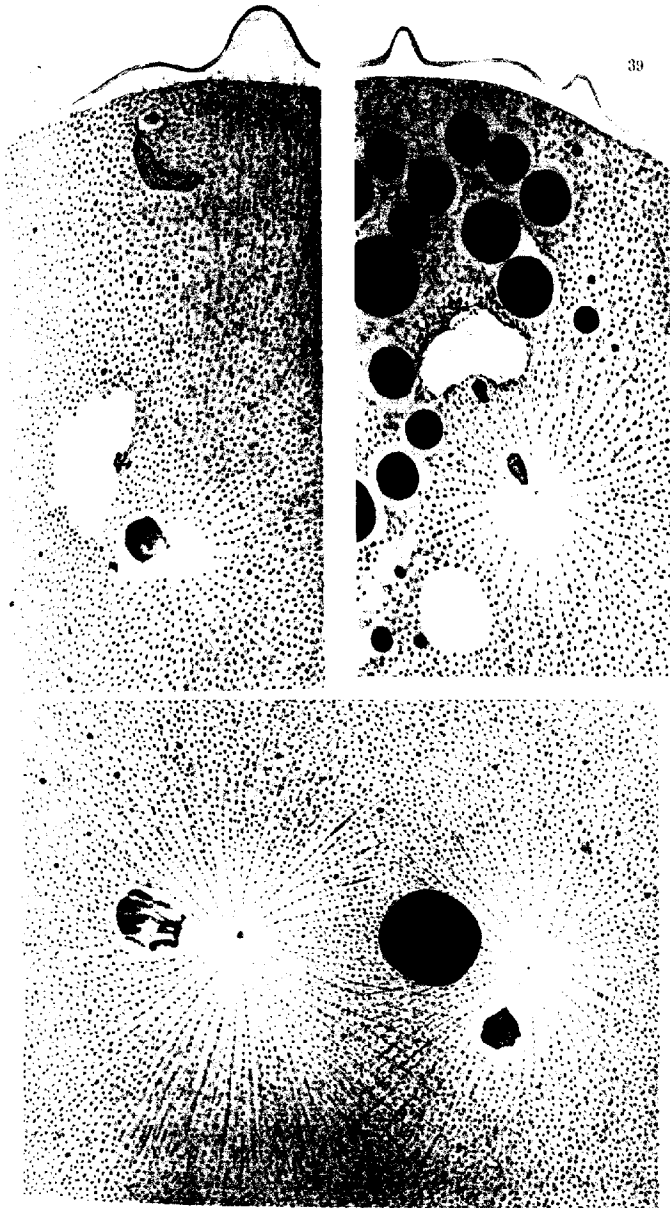


## PLATE 9

### EXPLANATION OF FIGURES

38 and 39 Two unusually small sperm nuclei with their asters, separating from the cones. From two eggs; compare the size of the entire sperm nucleus at this stage (fig. 11). History same as figs. 35 to 37.

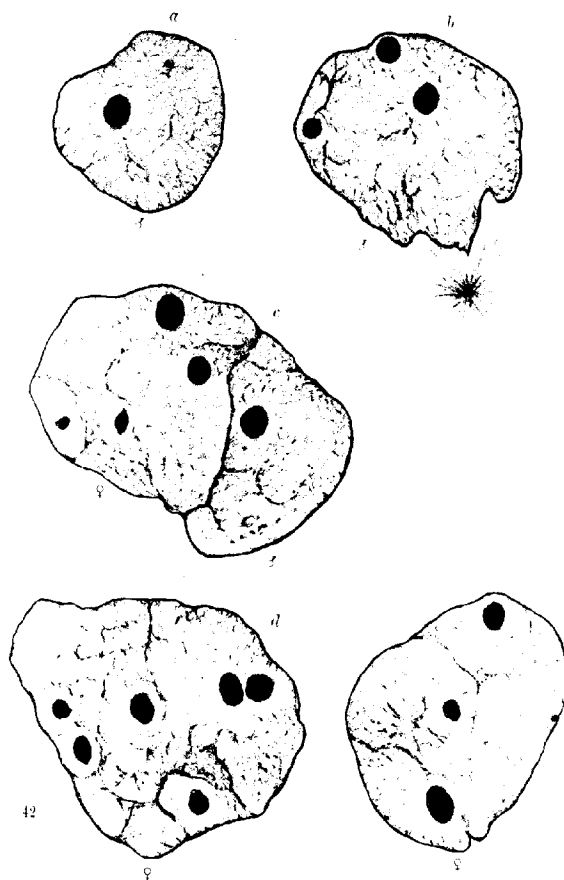
41 Two sperm nuclei of unequal size from the same egg. Compare size of centrosomes and asters. History: Centrifuged 7200 revolutions in thirty-six seconds, forty-four minutes after insemination; preserved ninety-two minutes after insemination. Reconstruction of three sections.



## PLATE 10

### EXPLANATION OF FIGURES

42 Five successive sections showing the entire germ nuclei of one egg. The male nucleus has five nucleoli, the female has thirteen. The line of apposition of the germ nuclei is seen in the third section. History: Centrifuged 7200 revolutions in thirty-six seconds, forty-four minutes after insemination. Preserved sixty-four minutes later.

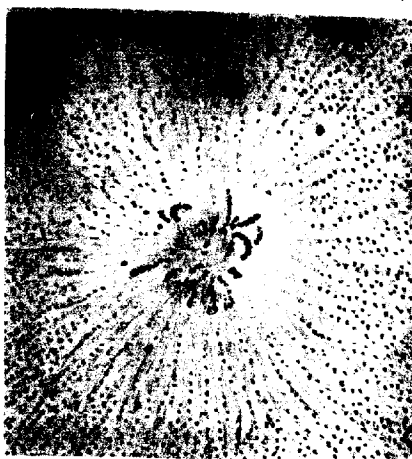


## PLATE 11

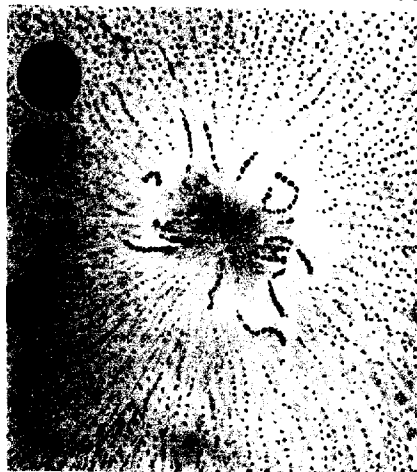
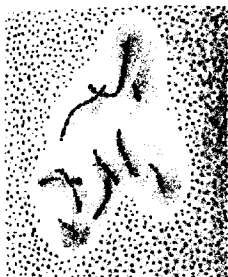
### EXPLANATION OF FIGURES

43 Three successive sections showing the entire egg nucleus of an egg from which the spermatozoön was entirely removed by centrifuging. Both polar bodies formed. Fourteen chromosomes indicated. History: Centrifuged 7200 revolutions in thirty-seven seconds, forty-two minutes after insemination. Preserved sixty-five minutes later.

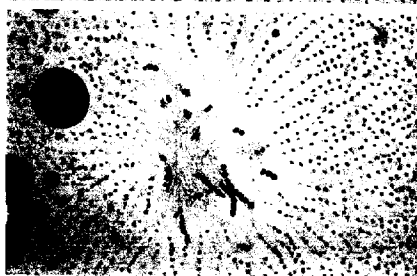
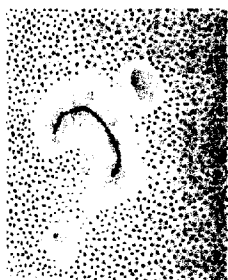
44 Three successive sections showing the entire egg nucleus of an egg from which the spermatozoön was entirely removed by centrifuging. The first polar body was not formed in this case, and a monaster arises around the egg-chromosome group. History same as fig. 43.



44a



44b



44c





# THE ELIMINATION OF THE SEX CHROMOSOMES FROM THE MALE-PRODUCING EGGS OF PHYLLOXERANS

T. H. MORGAN

*From the Zoölogical Laboratory, Columbia University*

TWENTY-NINE FIGURES

My studies of the life cycle of the phylloxerans of the hickories have shown first ('08) why the fertilized eggs produce only females,<sup>1</sup> and second ('09) that the production of the males is caused by the elimination of a chromosome from the male-producing egg.<sup>2</sup> One essential point in the life cycle still remained unexplained; namely, the cause of the production of small male- and large female-producing eggs. The differentiation of these two kinds of eggs precedes, in the life cycle, the formation of the true males and sexual females. *It may appear therefore that the question of the sex determination antedates those changes that lead to the elimination of a chromosome from the male-producing egg, and, if so, the real question of sex determination might seem to lie deeper than the manœuvres of the sex chromosomes.* Until this point is cleared up the value of the chromosome hypothesis in sex determination may seem to hang in the balance.

I am now able to bring forward certain evidence which I believe throws light on this important topic and I am prepared to offer an hypothesis based on the new evidence, which, if true, substantiates the view that one of the essential changes in the formation of the large and the small eggs is connected with changes in the sex chromosomes.

<sup>1</sup> Proc. Soc. Exp. Biology and Medicine, vol. 5, 1908, and Science, vol. 29, 1909.

<sup>2</sup> Proc. Soc. Exp. Biology and Medicine, vol. 7, 1910.

The main points that were described in my previous papers may be summarized as follows:

1. Two classes of sperm are produced in the male differing in the presence and absence of a pair of chromosomes. One class of sperm degenerates. It corresponds to the male-producing class of other insects. The other class produces functional spermatozoa, which entering the egg give rise to females only. These sperm correspond to the female-producing class of other insects.

2. The male-producing egg contains one less chromosome after the extrusion of its single polar body than it contained before this event. In a preliminary note ('10) I have stated how this elimination takes place and in the present paper I bring forward the evidence on which this statement was based.

3. The difference in size between the male-producing and the female-producing egg, before the former has extruded its polar body, proves that the predetermination of the males antedates the extrusion of the chromosome in the polar body of the (smaller) male-producing egg.

4. More male-producing individuals are the descendants of each stem-mother than female-producing individuals. The stem-mother must give rise to two kinds of eggs, i. e., they must be different either before or after the polar body is extruded. The factor that differentiates these two kinds of eggs, was not discovered. It is this point that the evidence now brought forward may I hope help to elucidate.

THE DIFFERENCES IN THE CHROMOSOME GROUPS IN THE POLAR  
SPINDLES OF THE STEM-MOTHER'S EGG AND OF THE  
MALE AND FEMALE-PRODUCING EGGS

In my former paper ('09) I have figured ten equatorial plates of polar spindles of the eggs produced by the stem-mother. In all of these the sex chromosomes are of nearly the same size. In two other plates one chromosome is much smaller than the others, which is probably due to this chromosome having been cut by the knife. Failure to find the missing piece in the next section would not be significant, since it might be very difficult to find such a piece in the egg filled with yolk granules of about the same

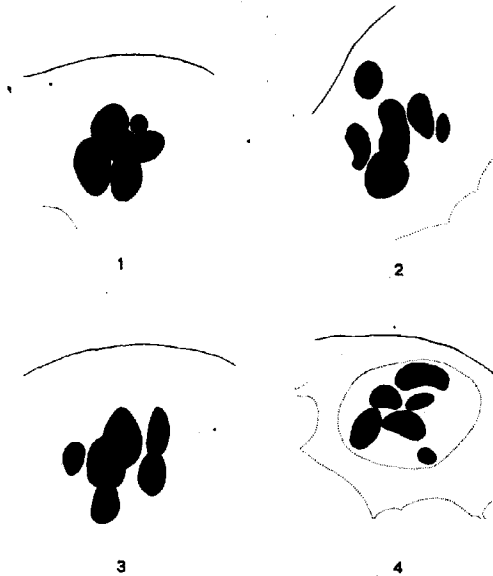
size as the chromosomes. Side views of the polar spindle, of which four are given, each with three chromosomes, show these to be of equal size. One of two cases of an egg nucleus (just prior to the formation of the spindle) also shows six equal chromosomes; the other case shows four equal chromosomes and one of double size that no doubt represents two chromosomes overlapping or else stuck together.

When these chromosome plates are compared with those of the female-producing egg shown in fig. LX, page 255 of my paper ('09), the size relations seem to be about the same. Only four plates of these eggs were found. I suspect that one of the equatorial plates that is assigned to a male-producing egg, namely, fig. LX; K, really belongs to a female-producing egg. Occasionally it is difficult, owing to the obliqueness of the sections, to make sure that a particular egg is a large or a small one. If this egg is excluded, or referred to the female-producing series, there remain fourteen equatorial plates of male-producing eggs. In all of them one chromosome is noticeably smaller than the rest. I can now add four more chromosome groups of male-producing eggs to this list, figs. 1, 2, 3, 4. Three of these are equatorial plates or just prior to that phase, and the fourth shows the chromosomes in the nucleus just prior to the spindle stage. They all contain five larger chromosomes, and one much smaller than the other five.

It is true that there is some variation in the relative size of the chromosomes in all of these figures, which makes it difficult to express exactly the relative sizes of the different chromosomes, and therefore I am aware of the danger of attempting to distinguish between the plates of the male- and female-producing eggs; yet the presence of the very small chromosome is so distinctive of the smaller eggs that I believe no error is committed in attributing to this difference at least a real significance.

If the two kinds of chromosomal groups just specified are significant one should expect to find a similar difference in the somatic cells of the individuals that give rise to these eggs, for since each of these individuals produces only one kind of egg (all the eggs found in one individual are male-producing or female-

producing) this difference should be apparent on inspection of the somatic cells of these individuals. In my paper I have, in fact, given nine plates taken from young stages of the development of the embryo. Some of these figures, notably fig. VII C, E, I, show six nearly equal chromosomes, while five of them, notably fig. VIII A, B, D, F, G show five larger and one smaller chromosomes. When these drawings were made the importance



of the size relations was not appreciated, and the number of cases is too small to be of great value, but it is significant, I think, that the two kinds of chromosome groups required by the hypothesis are actually represented in these figures.

It appears, then, probable that after the extrusion of the polar body of the egg of the stem-mother a change has taken place in those individuals that become the male-egg-producers. One chromosome has become smaller.

THE DIVISION OF THE POLAR SPINDLE IN THE MALE- AND  
IN THE FEMALE-PRODUCING EGGS

A brief abstract of the results given in this section was published in the Proceedings of the Society of Experimental Biology and Medicine<sup>3</sup> for May, 1910. In order to study the division of the polar spindle a large amount of new material was collected in the summer of 1909 which was cut and studied during the following winter. It has been most laborious to find eggs in which the polar spindle was in the process of division, and I wish to express my obligations to my assistant, Miss E. M. Wallace, who has found most of the new cases here figured.

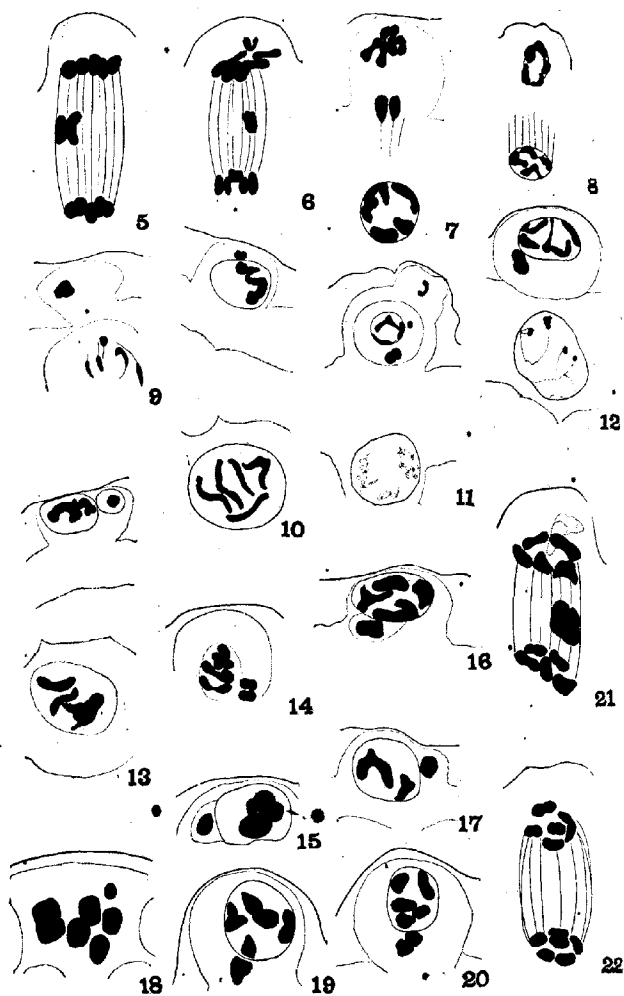
In my former paper ('09) I described the anaphase of two eggs that seemed to be female eggs (see below), but none of the male eggs; and it is the latter that would be expected to give the critical evidence. This evidence was briefly stated in my preliminary note in 1910: I shall now give drawings of several anaphases of male eggs that show beyond doubt that a lagging chromosome is present; that it passes to the outer pole, and forms a separate vesicle in the polar body.

The first case is shown in fig. 5 representing an anaphase of the polar spindle. Five chromosomes lie at the outer pole and five at the inner pole. In the middle of the spindle lies a double chromosome. It is relatively large and its two halves appear somewhat unequal. For reasons that appear later I shall speak of this as a single chromosome that has already divided into halves.

The second case is shown in fig. 6. Here also five chromosomes, somewhat elongated, lie at the outer pole and five at the inner pole of the spindle. In the middle of the spindle there is a double chromosome, its halves equal as far as can be determined.

The third case is shown in fig. 7. It represents a later stage; the polar body being in process of constricting from the egg. The group of chromosomes at the outer pole is now in process of division. Five chromosomes can be recognized, two dividing, and three having completed their division. At the inner pole

<sup>3</sup> Proc. Soc. Exp. Biology and Medicine, vol. 7, 1910.



the nucleus has begun to form. It contains five distinct chromosomes. Midway between the poles is the lagging chromosome completely divided into equal or nearly equal parts. A few traces of the spindle fibers are discernible. This is the clearest case that I have found and shows very distinctly the conditions at this stage.

The next case, fig. 8, is not so instructive, since the chromosomes in the inner nucleus have in one place seemingly stuck together so that only four bodies are seen. The lagging chromosome could not be found but the five outer chromosomes are distinct.

Fig. 9 shows the polar body nearly constricted off. The five inner chromosomes are clearly seen. The lagging chromosomes were not found and may have been fused with the lump of chromatin in the polar body that represents the massed chromosomes.

In fig. 10 (and in figs. 9, 11, 12 and 13) the egg nucleus is represented nearer the surface than in the actual section. The nucleus of the polar body contains a fused mass of chromatin. What appears to be the lagging chromosome lies on its outer wall, and is partially constricted into halves. The inner nucleus shows five equal or nearly equal chromosomes. As this is the only case observed where the lagging chromosome lies on the outer wall of the nucleus of the polar body, and as it is difficult to see how the chromosome could have reached this position; and moreover since the double body is smaller than the lagging chromosome in the other cases; it may be that this deeply staining body is not the lagging chromosome at all but a pair of displaced yolk granules. This interpretation is supported by the next case.

In this instance fig. 11 the inner nucleus is well formed and its chromosomes diffused or at least not stained. In the polar body there is a nucleus in which four chromosomes can be made out with the double lagging chromosome lying on the inner side of the nucleus. A yolk granule lies on the outer wall of the nucleus.

A similar stage is shown in the next figure, fig. 12. The inner or egg nucleus shows its contained chromosomes in process of



becoming diffuse. The polar body is cut off from the egg; its nucleus contains five chromosomes, and lying near the nucleus is the large lagging chromosome divided into two parts.

In the next figure, fig. 13, the chromosomes, both in the polar body and in the egg nucleus, have fused. The preservation may have been poor. The lagging chromosome lies in a vesicle of its own to one side of the polar body nucleus.

The polar body of another egg, fig. 14, shows five chromosomes in its nucleus, three of which at least are elongated as though dividing. The lagging chromosome lies outside. Its two halves are separated and each half is slightly dumb-bell shaped.

Three other polar bodies are shown in figs. 15, 16, and 17. Each shows the lagging chromosome outside of the nucleus; and in two cases surrounded by a partial vacuole of its own.

In addition to these new cases I have studied and redrawn some of the figures given in my former paper, restaining when necessary to better bring out the chromosomes. One of these, fig. 18, shows the equatorial plate of a male egg with one large chromosome (partly constricted), four intermediate, and one small chromosome. Two cases, figs. 19, 20, show polar bodies and their contained nucleus and the lagging chromosome outside. The third figure, fig. 21, shows the anaphase of an egg that I now interpret as a male egg. The interpretation of this anaphase figure is difficult because of the presence of a stained body near the center of the spindle. After staining and restaining several times it seems to me probable that this body is in reality a chromosome and not a yolk sphere as I formerly thought probable. Another sphere lies beyond the outer chromosome group and near it another body (in outline in the figure). Both of these seem to be in yolk spheres. At the inner pole five distinct chromosomes are present. If the stained body in the center of the spindle be interpreted as a chromosome the spindle bears a close resemblance to the spindle of the male-producing egg, but the egg is large, and mainly for this reason I was formerly inclined to think it a female egg. The case is doubtful and can not be interpreted with certainty.

I have tried to find again all the polar body stages figured in my former paper in order to reexamine them, but it has not been possible to discover several of them owing to the fading of the stain. I wished especially to reexamine figs. X; N, P, Q, which show the lagging chromosomes passing into the polar bodies, but have not succeeded in finding them again. All three represent the same spindle under different conditions. While these figures were drawn with as much care as possible I now realize that some of them might receive a clearer interpretation in the light of the information that I have gained from a new study of the polar spindle. One point is especially clear that in most cases the circle enclosing the chromosomes of the polar bodies of the male-producing egg was drawn so as to include the lagging chromosome, while in reality the ordinary chromosomes lie in one vesicle and the accessory in another clear area at the side of the latter. Thus in figs. X; C, G, I, L, M, R, S, T, U, the sixth chromosome generally shown as bifid represents the lagging chromosome.

In the new preparations I have found only one anaphase of a female-producing egg, fig. 22. Fortunately this is a very clear case. Six chromosomes lie at each pole, and there is no lagging chromosome present. Three of the outer chromosomes are dumb-bell shaped. The only other cases of this kind of egg are the doubtful case, here refigured, fig. 21, and the outer pole shown in my former paper in fig. X; K.

#### *Summary*

The evidence shows that four of the five chromosomes of the male-producing egg divide equally when the polar body is formed. One chromosome lies in the middle of the spindle and becomes divided into equal parts. It finally passes out into the polar body, just before the latter cuts off, and fails in consequence to become incorporated in the nucleus of the polar body. In all these respects its behavior is closely similar to that of the lagging chromosome described by McClung for this chromosome in the spermatocytes of orthoptera.

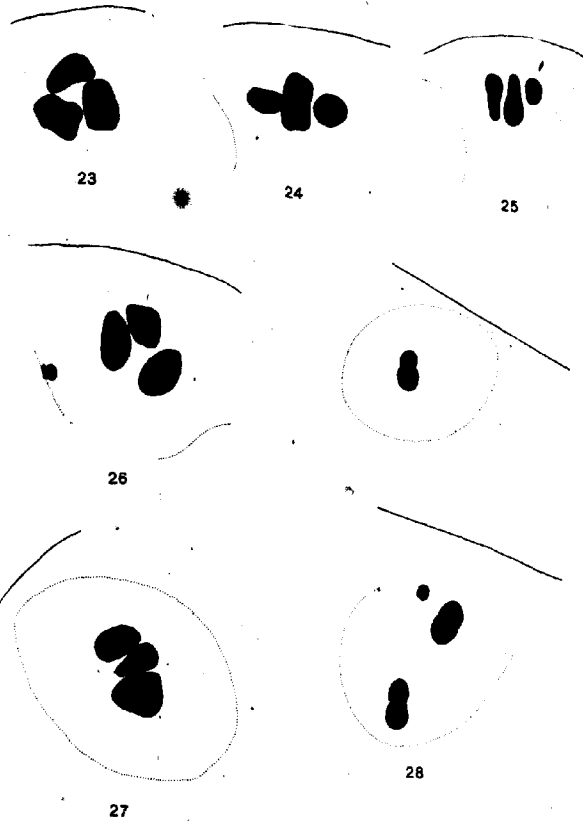
The six chromosomes of the female-producing egg appear to divide equally, so that the outer and the inner pole of the spindle get six chromosomes each.

One point of especial importance I have not been able to settle satisfactorily, namely, the fate of the smallest chromosome. Theoretically I should expect it to be the lagging chromosome. But the figures show the lagging as large as the rest. On the other hand it is equally clear that none of the others can be identified as the smallest—they appear to be of equal size. The evidence is therefore inconclusive either way. If the smallest is the lagging it must increase in size before it divides so that its size relations are changed.

#### THE POLAR SPINDLES IN THE SEXUAL EGG

Two questions of theoretical interest are involved in the anticipated reduction in the number of chromosomes in the sexual egg of the phylloxerans, namely, the number of the reduced chromosomes and their size relations. Whether the reduced number would be four or three could not be prophesied from the behavior of the chromosomes in the parthenogenetic series, for if, as I think, there are two small *x*-chromosomes attached to the two large *X*'s, the former might separate at the 'reduction period' to form a smaller pair, giving four chromosomes or else remaining attached to the larger *X* there would appear only three chromosomes. Six cases have been found showing clearly that the number of chromosomes in the sexual egg is three. I have found two eggs that show equatorial plates, figs. 23, 26; two eggs that show the chromosomes in the nucleus just before its resolution, figs. 27, 28; and two eggs that show side views figs. 24, 25. There can be no question that the reduced number is three. The size relations are more difficult to determine. In general it may be said that they are all of nearly the same size, although one of them generally appears larger than the other two. There is no such disparity in size between the largest and the smallest as that observed in the male-producing egg, which is a strong argument against my earlier suggestion that the smallest chromosome in the equatorial plate of the male-

producing egg is formed by the union of the two small  $x$ 's that leave their larger partners at this time and fuse in order that 'reduction' may occur. For, should this happen in the male-producing egg in order to insure the separation of the two small



$x$ 's one might anticipate a similar change in the sexual egg. We must conclude, therefore, that one of the pairs in the sexual egg represents the fusion of the two large  $X$ 's and their attached small  $x$ 's. One large  $X$  and one small  $x$  would therefore be left

in the egg. The female-producing sperm brings in during fertilization a large  $X$  and a small  $x$  which brings the number back to the six chromosomes (in reality eight since two are double,  $X$  and  $x$ ) of the stem-mother's soma.

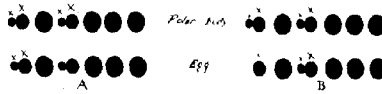
#### THEORETICAL INTERPRETATION OF THE RESULTS

From the results given in preceding sections I draw the following conclusions. The cells of the stem-mother (that comes from the fertilized egg) contain six equal or nearly equal chromosomes. Two of these, that I call  $X$ , have attached to them two smaller chromosomes that I call small  $x$ . The stem-mother's cells have therefore in reality eight chromosomes. The eggs produced by the stem-mother also contain these six (or eight) chromosomes that appear in the equatorial plate of the polar body. One polar body is extruded. The division of the chromosomes has not been observed. I assume that at this time all of the chromosomes divide equally, except in the case of those eggs that will become male-egg producers. In these eggs one of the small  $x$ 's passes undivided into the polar body. Presumably it passes out attached to the outgoing half of the larger  $X$ , with which it has been fused. Unless it separated from the large  $X$  it might not appear as a lagging chromosome at the time; if it became detached it might appear as a lagging chromosome; or both the large outgoing half of the large  $X$  with its attached small  $x$  might lag behind the rest. Further work will be necessary to settle this point. This kind of egg, after the polar body is extruded, will contain six chromosomes, one having been reduced in size by the loss of the small  $x$ . This group appears in the equatorial plate of the polar spindle of the small 'male' egg. The difference in the size relations of the chromosomes observed in the polar spindle of this egg, as compared with the size relations of the chromosomes in the stem-mother's egg is accounted for by the loss of one chromosome—the small  $x$ . If eight chromosomes are present in the stem-mother's cells and eggs there are only seven in the body cells and in the eggs of the male-producers. When the polar body of the male-producing egg is formed all the chromosomes divide except one, which, lagging on the spindle, finally passes

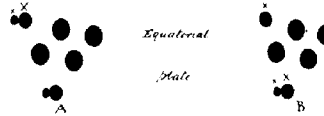
Chromosomes of stem  
mother's polar spindle



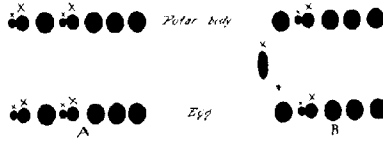
Two types (A and B) of division  
of above to produce the  
female line A and the male line B



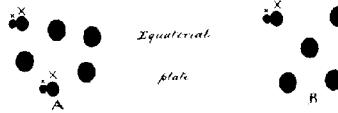
Polar spindle of female  
producing egg A; and male  
producing egg B



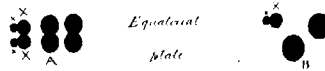
Division of last



Somatic cells of female  
A, and male B



Reduced number of  
chromosomes in sexual egg A,  
and in spermatocyte B



Division of Polar spindle  
in sexual egg A. First  
division of spermatocyte B

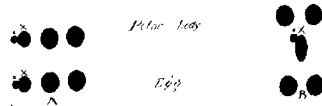


DIAGRAM 1

out bodily into the polar body dividing at this time as do also the members of the outer group of chromosomes. The result is that five visible chromosomes remain in the male egg, in reality six chromosomes, since the large  $X$  and the small  $x$  are attached to each other. In the body cells of the male these two  $X$ 's often remain united, but sometimes partially separate. When reduction takes place three visible chromosomes are present in the spermatocytes (one of these three is the fused pair). Two of these divide equally at the first division, and one (the fused pair) lags behind and finally passes to the female-producing sperm. Toward the end of this division the large  $X$  and the small  $x$ , not infrequently partially or even completely separate. In the second spermatocyte division all the chromosomes of the female-producing cell divide equally, giving rise to two functional sperm containing three visible chromosomes, or four in reality since one is double. The class of cells without the  $X$ 's degenerate.

Returning again to the stem-mother in order to trace the history of the female line I assume that when the polar spindle divides all of the eight chromosomes divide, leaving eight in the egg (six visible) of which there are two pairs, each containing a large  $X$  and its attached small  $x$ . The eggs develop into the female producers, whose polar spindles contain six equal chromosomes like those of the polar spindle of the stem-mother. The larger number of chromosomes in the female-producing egg accounts for the larger size of the egg, as compared with the male-producing egg, which, as shown above, contains one less chromosome (the small  $x$ ). When the polar body is set free the chromosomes divide equally, six (in reality eight) passing out, and six (or eight) remaining in the egg. The body cells of the sexual female contain therefore six (eight) chromosomes. A reduction division occurs in the sexual eggs, so that three (in reality four) chromosomes appear. When the polar bodies are formed—there are two of them to judge by analogy with the aphids—three whole chromosomes (in reality four) are given off at one of the divisions so that three (in reality four) remain in the egg. The female-producing spermatozoon introduces into this egg during fertili-

zation the same chromosome group, which brings back the number of chromosomes to that characteristic of the stem-mother, and starts the same cycle again in the next year.

The attachment of the two small  $x$ 's to the two large  $X$ 's, that is assumed to occur throughout this series, except when one of the small  $x$ 's is supposedly lost in the male-producing line of the stem-mother's egg, and the loss of one large  $X$  when the male egg extrudes its polar body, may seem to be the most doubtful points in the preceding account. That a small  $x$  is actually present is shown clearly in the spermatogenesis, and in some of the somatic cells of the male. It is, therefore, highly probable that the other large  $X$ , found in the stem-mother and female line, has also a small  $x$  attached. Otherwise a symmetrical distribution of the chromosomes can not take place. But my assumption that one small  $x$  is eliminated from those eggs of the stem-mother that give rise to the male line may appear more problematical. I readily grant that this is hypothetical. There are two facts, however, that give the hypothesis some probability. First, by means of this hypothesis the change in observed size-relations that takes place in the chromosome group of the male-producing egg can be accounted for. Second, the apparent absence of the small chromosome, in the lagging chromosome of the polar spindle of the male-producing egg, supports this view. On the basis of these two observations I have ventured to offer the above hypothesis, especially as it seems to give a consistent view of the changes that take place at the most critical stage in the life cycle when two lines are produced. In my former paper I have pointed out that there is no external condition that appears adequate to account for this dichotomy, and, if this is correct, we are warranted in looking for an internal factor that produces the result. The assumption moreover is in accord with the view, now well established, that the production of males is associated with the absence of certain chromatin in the egg. From this point of view the male-egg-producer—the winged migrant—is half a step towards the production of a male; the final step is taken when the other  $X$  is eliminated, which demonstrably occurs at the next stage when the polar body of the male egg is eliminated.



That an  $X$  chromosome may be present attached to another chromosome has been shown in recent years by Boveri, Boring and Gulick in several species of *Ascaris*. The same looseness of attachment that I have observed has also been found in the eggs of *Ascaris* where in certain individuals and at certain times a separation has been recorded, while in other individuals the  $X$  chromosome remains completely united with its larger companion. In my own case the attachment is between two  $X$  chromosomes while in the other cases the attachment is between an  $X$  and what is apparently an ordinary chromosome.

#### OTHER POSSIBLE INTERPRETATIONS CONSIDERED

In my former paper when dealing with the differences in the chromosomal groups in the equatorial plates of the polar spindles of the male- and female-producing eggs I have suggested that the change could be accounted for if in the male egg the two smallest chromosomes (the two small  $x$ 's) each left its larger partner and fused together while the two larger also fused. This would give the same actual number as before, but a relative difference in size would result. The logical conclusion from the assumption would be that when the polar bodies are given off the large  $X$ 's separate (reduction division for the pair) and the small  $x$ 's also separate, the other chromosomes dividing equationally. This assumption was necessary for the large  $X$  and the small  $x$  pair because in the spermatogenesis a large  $X$  and a small  $x$  are still present.

If this view were correct the lagging chromosomes in the anaphase of the male egg should consist of a large and a small chromosome. The facts show that two chromosomes do actually lag but unfortunately for the assumption they are equal and relatively large. The doubleness of this lagging body can be better explained by a precocious division into equal parts, since the other chromosomes that pass out into the polar body also show signs of division at this time. It is clear that my former interpretation must be abandoned.

There is another interpretation that might be considered, namely, that the lagging body is really a large  $X$  and small  $x$  closely united

and the division is equal in both as in the other chromosomes that pass out. This would leave a similar pair (large  $X$  and small  $x$ ) in the male-producing egg which becomes the lagging pair in the spermatogenesis. This hypothesis works out consistently but it leaves unexplained the observed size differences that appear in the chromosome groups of the male egg; it also leaves unaccounted for the production of the smaller male egg and it 'explains away' the observed size relations in the lagging chromosomes of the male egg. Hence I think this view must also be put aside.

Again we might assume that the large  $X$  is the sex chromosome and the small chromosome attached to it (its synaptic mate) is in reality not an  $X$  at all, but a  $Y$  chromosome. Were this the case the  $Y$  should pass into the male-producing sperm since this is the characteristic behavior of  $Y$  in other insects. As it does not do so there is no basis of fact to support such an interpretation.

Lastly, one may ask whether the two large  $X$ 's in the stem-mother's egg are of the same size and also whether the two small  $x$ 's present are of the same size. Assume for instance that the two smaller  $x$ 's are unequal in size. If the larger of them should pass out into the polar body of the stem-mother's egg the egg might become a male-egg-producer, if the smaller passes out the egg might become a female-egg-producer. In this way the two lines become differentiated. But this would leave in the female line two  $X$ 's and one small  $x$  (the larger one). We should have to assume then that the sexual egg eliminates one large  $X$  and retains the other large  $X$  and the other  $x$  (its companion). In other words a second differential division must be assumed. In the absence of evidence we are scarcely justified in making two such assumptions. Moreover, if this view were correct we should expect to find a chromosome group in the polar spindle of the female producing egg like that in the male-producing egg, but this is what we do not find. Of course if the larger of the small  $x$ 's that is assumed to be left in the female-egg-producer were much larger than that left in the male-egg-producer the size differences might not be so marked, but until this can be established we are

scarcely justified in making this assumption even although by doing so we may seem to give an attractive explanation of the splitting into the male and female lines.

Other combinations will suggest themselves but offer no advantages I believe over the one that I have suggested.

COMPARISON BETWEEN PHYLLOXERA CARYAECALIS AND  
PHYLLOXERA FALLAX

It may be worth while to compare briefly the conclusions reached for *P. caryaecalis* with the results described in my former paper for another species, *P. fallax*. The latter has twelve chromosomes in the equatorial plate of the egg laid by the stem-mother. After the extrusion of the polar bodies I have described twelve chromosomes as the number for the cells of the embryo. If as in *P. caryaecalis* there are two pairs of *X*'s present that are united this number would still be found, but if this view were correct for the male line we should expect to find six in the male spermatocytes of which one is double. Five should divide equally and the double one should lag with or without showing its doubleness at this time. In reality only four divide equally and two whole chromosomes that were separate in the equatorial plate of the spermatocyte come near together and become the lagging chromosomes of this division. There can be no question of their relations in the spermatocytes since the chromosomes are perfectly distinct and hundreds of such stages have been studied. It seems necessary therefore to recast this first view and to re-examine the facts. In fig. II; *A-F* of my paper six chromosome groups of polar spindles of male- and female-producing eggs are drawn. Of these groups three show twelve chromosomes, two show eleven, and one is doubtful. In fig. II a; *C-T* there are nine groups showing twelve chromosomes, one is doubtfully twelve or eleven, one shows eleven, and one shows ten. These are all from winged individuals. These retain their eggs for some days and several ripe eggs are found in the body of each individual while the wingless individuals, which have replaced largely the winged in this species, bring to maturity only one egg at a time which is laid as soon as it is ripe. It is therefore more difficult

to get polar body spindles in this type. The winged individuals produce only male eggs, so far as I have found. Therefore the fifteen equatorial plates of the winged migrants supposedly belong to male eggs. Now it is well recognized that the large number of chromosomes is more likely to represent the typical number, for, when one or two are lacking they may be in other sections, or be cut, or obscured by the other chromosomes. It appears then that twelve chromosomes are present in the male eggs.

I found only two cases in wingless individuals in which the number of chromosomes could be clearly counted, fig. II a U and W. In both ten chromosomes are present, but two are of double size and probably represent the four X's fused together in two pairs. There is no *visible* evidence therefore of the loss of one chromosome in the eggs that give rise to the male-producers as I have postulated for *P. caryacaulis*.

After the polar bodies have been given off from the large and the small eggs—unfortunately I have never found a spindle in process of division—two kinds of embryos are found, namely, those containing twelve and those containing ten chromosomes. It appears therefore that two chromosomes are lost from the smaller egg. Moreover only ten chromosomes appear in the spermatogonial cells. The evidence therefore may seem to point to the conclusion that in this species there is usually no loss of chromosomes in any of the eggs (at least none that can be pointed out) of the stem-mother at the time when the polar bodies are formed. Hence the separation into the male-producers and the female-producers at this time can not be shown to be due to the loss of one of the twelve visible chromosomes.

It may appear therefore that the evidence contradicts the hypothesis offered to account for the change in the other species, but it will be noticed that the comparison rests in the assumption that the two equal X chromosomes in the spermatocytes of *P. fallax* correspond to the large X and the small x of *P. caryacaulis*. But it may equally well be true that there are two large X's with two small x's attached (that do not become visible) in *P. fallax*. In other words not only are there twice as many ordinary chromosomes but twice as many sex chromosomes also. The

total number of one species is double that of the other. If this is the case the loss of the two smaller  $x$ 's might take place in those eggs of the stem-mother that give rise to the male producers and its loss might not be apparent unless its mate were sufficiently reduced in size to make its loss visible. It has not been possible to make out the size relations of the chromosomes in *P. fallax* with sufficient clearness for the evidence to be of any value one way or the other. The question for this species must be left unanswered, but it should at least be noticed that the two large lagging chromosomes of *P. fallax* behave like the single lagging chromosomes of *P. caryaecaulis*, and not like the large  $X$  and the small one of the latter form.

## AN EXPERIMENT DEALING WITH SEX-LINKAGE IN FOWLS

A. H. STURTEVANT

*From the Zoological Laboratory, Columbia University*

FOUR FIGURES

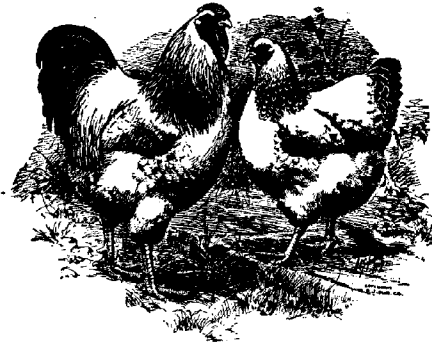
In 1911 I published a preliminary report (Sturtevant, '11) of an experiment showing sex-linked inheritance in fowls. The more significant data for the second generation can now be given.

### DESCRIPTION OF PARENT BREEDS

The two breeds used in this experiment were the Columbian Wyandotte and the Brown Leghorn. The Columbian Wyandotte (fig. 1) is chiefly white, but with the tail, primaries, upper web of secondaries, median stripe to neck feathers, and some tail coverts black, in both sexes.

The Brown Leghorn is nearly the color of the wild *Gallus bankiva*.<sup>1</sup> The sexes are strongly dimorphic. The males have reddish yellow edging to the neck and back feathers, some red on the shoulders and wing coverts, and a rather yellowish brown lower web to the secondaries (wing-bay of the fanciers), the rest of the plumage being typically black. The female has the tail, primaries, upper web to secondaries, and stripe in neck feathers black. Her breast is salmon yellow, and the neck feathers are edged with yellow. The rest of the plumage is finely stippled or mottled with yellowish brown and black.

<sup>1</sup>A very good illustration showing male and female of this color is given by Bateson ('09, pl. 4, figs. 3-4, opp. p. 103).



## DESCRIPTION OF CROSS-BRED BIRDS

In  $F_1$  the males are all alike, whichever way the cross is made. They are of fairly typical Columbian color, but perhaps rather darker than the parent stock, showing black stripes in some back feathers and black ticking (small specks) elsewhere. These characters, however, may occasionally appear in a pure Columbian male. There are two types of  $F_1$  females, depending on the direction of the cross. When a Columbian male is used they approach the Columbian color, but differ in having coarse irregular stippling of the Leghorn type, in the same places as in the Leghorn female. These and the cross-bred males of the type described above I shall, for convenience, call grays. The  $F_1$  red females obtained from the Brown Leghorn male and Columbian Wyandotte female (called browns in my earlier paper) are simply grays with the white replaced by a uniform red, about the shade of the breed known as Rhode Island Red. The  $F_2$  results have shown that they are not to be considered as browns, genotypically like the Brown Leghorns, as I had supposed they were.

In  $F_2$  occurred several new types, which I shall describe before proceeding to the analysis. Red males, like red females, are simply grays with red substituted for white.

Another quite new type of males occurred—the duckwing. This type has the Leghorn or Jungle fowl pattern, with black-striped straw-colored neck and saddle, white wing-bay, red shoulders and back, black primaries, tail, and wing coverts, and mainly black breast, though a little white and some brown shows here. Now pure Brown Leghorn males occasionally show some brown on the breast, and some of the white on the only specimen of the above class examined in adult plumage is probably due to the fact that he was sick when young, and did not grow well (but see letter from Mr. Westfall quoted below). Therefore I believe this male is a brown with white or straw-white substituted for red and yellow in neck, saddle, and wing-bay.

There are two types of duckwing females. One, the silver gray, is the color of the Brown Leghorn female, with all the brown or yellow except that of the breast, replaced by white or light gray. This replacement is, however, incomplete on the back and wings. This is the color of the Silver Duckwing Game or Leghorn, and of the Silver Gray Dorking. I have seen brown in the above mentioned regions on exhibition specimens of two of these breeds. What I have called the brown duckwing female has the yellow replaced by white only in the neck. I know of no breed where this condition appears (but see statement in letter from Mr. Westfall, quoted below.)

The presence of these two types of females suggests that there should be two types of duckwing males. Perhaps one type should be like the Silver Gray Dorking or the Dark Brahma males, which have the Brown Leghorn color with all brown and red replaced by white. Or perhaps there should have been golden duckwings, that is some with straw-colored wing-bay, as well as neck, back, saddle, and wing-bow. I had five duckwing males, but raised only one to maturity. The last time I examined the others they were not quite three months old. At that time all five looked about alike in color, and my notes regarding them are as follows: necks black and white; backs red, slightly stippled with black, straw-colored saddle feathers begin-



ning to appear; wing-bows red and black; coverts and wing-bays brown stippled; breasts and tails black. In this connection it may be of interest to note what Mr. Henry Hales, breeder of Silver Gray Dorkings, says (in a letter dated December 28, 1911) in regard to the juvenile plumage of males in that breed:

The males have not the stippling like the females at any age: before adult feathering the cockerels have the coloring mixed up with white, black, and brown. One would hardly believe they would have the decided colors of the full plumage of the cocks. One cannot judge what the young ones are going to be until they are fully feathered.

In view of this statement, it may be that my duckwing male was not far enough advanced when killed to show quite the adult plumage. Mr. Watson Westfall, another breeder of Silver Gray Dorkings, wrote to me, under date of December 29, 1911, giving a somewhat different statement, as follows:

The Silver Gray female was once quite a brown colored hen and the males did not have white saddles and hackles but were straw color with some red across the shoulders. At this time they were known as Gray Dorkings, but by continual selection the English bred this brown out of them, and when the saddle, back, and hackle of the male was quite free of red and straw color and the female more gray the word Silver was added, making the name Silver Gray as we have it now. As the chicks hatch now with the very pure white top male as sire all the cockerels show themselves plainly at once by being whiter on the head than the pullets, but as soon as they begin to feather they soon become very closely alike and remain so until the adult feathers appear, when the males will show black on breast while the females will begin to show red. But like all such varied colored fowls the female gets her real color long before the male. At two months old and up until the time the adult feathers begin to show on the cockerels they have a lot of stippled feathers on wings and back, but not many are as evenly stippled as the pullets, and where they are so much stippled and there is no brown at all to show on the wings, such specimens are very apt to be splashed with white on the breast. The red doesn't all want to be lost in the chick feathers or else there will be failure in breast coloring.

Specimens representing the grays (one ♂ and one ♀), reds (one ♂), duckwings (one ♂ and one ♀), and extracted browns (one ♀) are deposited with the Zoölogical Laboratory of Columbia University.

The matings made and the offspring produced were as follows:

I. Columbian Wyandotte ♂ × Brown Leghorn ♀	→	9 gray ♂ 3 gray ♀
II. Brown Leghorn ♂ × Columbian Wyandotte ♀	→	10 gray ♂ 8 red ♀
III. Brown Leghorn ♂ × Red F <sub>1</sub> ♀	→	3 red ♂ 3 red ♀
IV. Gray F <sub>1</sub> ♂ (from II) × Brown Leghorn ♀	→	3 gray ♂ 5 duckwing ♂ 5 red ♂ 7 gray ♀ 1 silver gray ♀ 1 brown duckwing ♀ 2 red ♀ 2 brown ♀

#### EXPLANATION OF RESULTS

As I pointed out in my preliminary note, there is here at least one sex-linked factor, which I then called *G*, causing the two types of F<sub>1</sub> females. The F<sub>2</sub> generation agrees with this, mating III giving no grays, and mating IV both gray and non-gray males and females. But there are obviously other factors contributing to the F<sub>2</sub> result, which is decidedly complex, as has so often been found to be the case in experiments with fowls. I do not, therefore, feel justified in giving more than a tentative explanation of the results, since the numbers are small, and only a few of the many crosses which would be required to test any explanation have been made. The following will, however, cover the results obtained, and is the simplest scheme that I have been able to work out.

Let us assume that the Columbian Wyandotte carries an inhibitor, *I*, for red in all parts of the body, with the exceptions noted below. This is the *G* of my earlier paper. The Wyandotte also carries another sex-linked inhibitor, *N*, which prevents the production of red in the neck (and saddle of the male), not affecting the other parts. This is probably the factor described by Davenport ('11) as found in Dark Brahmas. Birds not carry-

ing these two factors have these regions of a red or reddish color, so that the Brown Leghorn, and probably also the Columbian Wyandotte, must carry a factor for red, *R*. The factors *I* and *N* do not completely inhibit *R*, since most of my *I*-bearing birds show traces of brown here and there, and all the white necked males are very 'brassy' (yellowish). Both these characters sometimes appear in pure Columbian Wyandottes. Apparently there is also a Leghorn pattern factor, *L*,<sup>2</sup> causing black breast and black on the wing coverts in the male, and black stippling and salmon breast in the female, the latter effect appearing even in the presence of *I*. The factor *L* is hypostatic to another pattern factor, *P*, which is carried by the Columbian Wyandotte, and which inhibits all the colors just mentioned, as caused by *L*, leaving the color of the part dependent upon *R* and its inhibitors. But one dose does not completely inhibit the stippling of the female.

An alternative view, equally as satisfactory, I think, is that there is no inhibitor *P*, but that the Wyandotte has no *L*, and that the absence of this factor is dominant to its presence, heterozygous females being distinguishable by the stippling. On the first view *L* is probably present in all my birds. The constitution of the various types would then be as follows:

Columbian Wyandotte	INRPL
Brown Leghorn	inRpL
Gray	INRPL or InRPL
Red	inRPL
Silver gray	INRpL, or InRpL
Brown duckwing	iNRpL

One other combination is possible—iNRPL. This should give a bird with Columbian pattern, white or straw neck, and red body. It is possible that such would have appeared had more birds been raised, but I know of no variety having any similar color combination, and have never observed it in a cross-bred

<sup>2</sup>This is probably one of the components of the *J* (Jungle pattern factor) of Davenport ('09).

fowl. This seems to me to be one of the most difficult points in connection with the hypothesis here given to explain my results. It may be that there is some interaction between  $N$  and  $P$ , such that when both are present  $N$  cannot produce its effect. Then INRPL would give red. This could be tested by raising large enough numbers from mating IV to find out the real  $F_2$  proportions, or by testing a number of reds with Brown Leghorns and seeing if any of them gave brown duckwings in  $F_1$  or  $F_2$ . My principal evidence indicating that  $N$  is sex-linked was the fact that the females from mating II had red necks. But since they also had  $P$ , if the above hypothesis is correct, the only good reason for making  $N$  sex-linked is that it is probably identical with the factor described by Davenport ('11) as being sex-linked. If it is not so linked, then some of the reds from mating III should also carry it, and that mating should, eventually, produce some brown duckwings.<sup>3</sup>

Since I have no evidence that  $R$  or  $L$  is missing in any of my birds I shall simplify the following formulae by omitting them. In these formulae  $MM$  represents a male,  $Mm$  a female.

I. Columbian Wyandotte ♂	INPM	INPM	
Brown Leghorn ♀	inpM	inpM	
	INPM	inpM	-- gray ♂
	INPM	inpM	-- gray ♀
II. Brown Leghorn ♂	inpM	inpM	
Columbian Wyandotte ♀	INPM	inPm	
	inpM	INPM	-- gray ♂
	inpM	inPm	-- red ♀
III. Brown Leghorn ♂	inpM	inpM	
Red ♀ (gametes)	inPM	inpM inPm inpM	
	inpM	inPM	-- red ♂
	inpM	inpM	-- brown ♂ (not seen)
	inpM	inPm	-- red ♀
	inpM	inpM	-- brown ♀ (not seen)

<sup>3</sup> The real solution of this difficulty may be that  $L$  and  $N$  are coupled.

IV. Gray ♂ (gametes) Brown Leghorn ♀	INPM inpM	INpM inpm	INPM INpM InPM InpMiNPM iNpM inPM inpM
	INPM inpM	— gray ♂	
	INpM inpM	— duckwing ♂	
	InPM inpM	— gray ♂	
	InpM inpM	— duckwing ♂ (silver gray)	
	iNPM inpM	— red ♂ (white-necked?)	
	iNpM inpM	— duckwing ♂	
	inPM inpM	— red ♂	
	inpM inpM	— brown ♂ (not seen)	
	INPM inpm	— gray ♀	
	INpM inpm	— silver gray ♀	
	InPM inpm	— gray ♀	
	InpM inpm	— silver gray ♀	
	iNPM inpm	— red ♀ (white-necked?)	
	iNpM inpm	— brown duckwing ♀	
	inPM inpm	— red ♀	
	inpM inpm	— brown ♀	

## OTHER EXPERIMENTS DEALING WITH THE SAME COLORS

Bateson ('02, '09) and Punnett ('05) have given some facts regarding the duckwing color. When the Brown Leghorn was crossed with the White Dorking or White Leghorn, they obtained in F<sub>2</sub> some silver gray females. Two of these mated to a pure Silver Gray Dorking male gave only silver grays, and of these, four females to a male gave only silver grays. From these facts Bateson ('09) infers that the replaced red and yellow of the Brown Leghorn probably depends upon a separate factor, which his white breeds lacked. It seems to me more probable that this factor, which I have called *R*, was present in all three breeds, and that the two white breeds carried also the factor *I*. Any F<sub>2</sub> female showing the silver gray color would then be as pure for *I* as a pure Dorking, the factor being sex-linked, which explains why they had no trouble in getting a dominant F<sub>2</sub> to breed true.

Mr. T. Reid Parrish, a Columbian Wyandotte breeder, has published in advertising circulars and in poultry journals (e.g., Parrish, '11) detailed accounts of how he originated a strain of Columbian Wyandottes (probably not the one used in my experiments). According to this account he used Light Brahma

females with a White Wyandotte male. The Light Brahma has exactly the color of the Columbian Wyandotte. The breed seems to have been brought from the Orient in something like its present form, so that its history as to the origin of its color must probably remain a matter of conjecture. The White Wyandotte was derived directly from the Silver Laced Wyandotte, and is still, or was comparatively recently, a not uncommon sport from that variety (see McGrew, '01, and poultry literature generally). This would seem to indicate that it is a recessive white, probably due to the dropping out of a color producer. Mr. Parrish's statements support this view, as he says he obtained, in the  $F_1$  generation from his cross, silver laced, barred, and Columbian birds—apparently no whites. These  $F_1$  silvers he says were not typical, some of them having nearly white breasts, "yet showing a trace of lacing throughout the plumage." This sounds as though they were much like the birds I obtained in my cross between silvers and Columbians (Sturtevant, '11). From the result of that cross it would appear that silver is incompletely hypostatic to Columbian. Mr. Parrish says of his  $F_1$  barred birds mentioned above that they "showed stronger Brahma markings than the silvers, but there was unmistakable barring throughout the plumage, being especially noticeable in tail and wing, some specimens showing barring in every section." This is a most interesting statement, in view of the work of Spillman ('09), Goodale ('09, '10), Pearl and Surface ('10), and Davenport ('06, '09) on barring. In this connection it is worth noting that occasionally a few barred feathers occur in pure Columbian Wyandottes, especially in the tail coverts of young males, and that one of my  $F_2$  males (a duckwing) in the experiment described above has some barring in his hackle.

Mr. Parrish states that he mated his  $F_1$  Columbians with White Wyandottes, reciprocally. From Columbian female he obtained the same three classes as in  $F_1$ , but we are not told whether or not whites appeared. The mating with Columbian male gave whites and Columbians, but he doesn't say what else, if anything.

The only conclusions which I feel safe in drawing from these data are that the White Wyandotte is a recessive white, lacking a color producer, and that it carries a silver laced determiner. Bateson ('02) gives confirmatory evidence for the first of these conclusions.

#### SEX FORMULAE

It will be noted that I have used above the  $MM$ ,  $Mm$  scheme for sex formulae in preference to the more usual  $Ff$ ,  $ff$  formula (see Morgan, '11). I have made the change because the formula used here gives a mechanism which allows both complete sex-linkage, and also incomplete association with the sex-determiner. I shall now present the evidence which has led to this view.

It seems to me that the evidence now before us warrants the conception of the chromosomes as the carriers of Mendelian factors or genes, as a working hypothesis. This conception is especially helpful in considerations of sex-linkage and the other forms of gametic coupling or associative inheritance. The recent hypothesis put forward by Morgan ('11 a, '11 b, '11 c) to explain these phenomena seems to me to overcome the old difficulties encountered by the chromosome hypothesis of Mendelian inheritance. I shall make this conception the basis of my argument in favor of the  $MM$ ,  $Mm$  scheme.

Sex-linked inheritance of the type concerned here has now been known for some time, and has been recognized in Lepidoptera and in birds, as follows: Abraxas (Doncaster and Raynor, '06; Doncaster, '08), canaries (Durham and Marryatt, '08), fowls (Bateson, '09; Spillman, '09; Goodale, '09, '10; Pearl and Surface, '10; Bateson and Punnett, '11; Davenport, '11; Sturtevant, '11), and ducks (Goodale, '11).

Since my argument for the  $MM$ ,  $Mm$  sex formula depends largely upon certain cases which I believe to represent partial sex-linkage, it will perhaps be well to present in some detail the evidence for the existence of this phenomenon. In this category I have included three cases of the Abraxas type (one in the fowl, one in the canary, and one in *Agria tau*), and one, which I shall describe later, in the *Drosophila* type of sex-linkage.

Bateson and Punnett ('11) describe certain exceptions occurring in their sex-linkage experiment with fowls, which they suggest may be due to a failure of the usual association between the sex-linked factor and the sex-determiner, i.e., to 'crossing over' in the female. This is what I mean by partial sex-linkage.

The sex-linked factor in canaries transforms pink eyes to black, and may then be represented by the symbol *B*. The following crosses have been reported by Durham and Marryatt ('08):

Black ♂	BM BM
Pink ♀	bM bm
	BMbm — black ♂ 19
	BMbm — black ♀ 7

F <sub>1</sub> black ♂	BM bM
Black ♀	BM bm
	BM BM — black ♂ 24
	BM bM — black ♂ 24
	BMbm — black ♀ 18
	bM bm — pink ♀ 13

F <sub>1</sub> black ♂	BM bM
Pink ♀	bM bm
	BMbm — black ♂ 24
	bM bM — pink ♂ 21
	BMbm — black ♀ 5
	bM bm — pink ♀ 19

Pink ♂	bM bM
F <sub>1</sub> black ♀	BM bm
	bM BM — black ♂ 4
	bM bm — pink ♀ 6

It will be seen that all the above crosses give the typical Abraxas results if *B* and *M* be assumed to be completely coupled,<sup>4</sup> but I have purposely omitted one cross:

<sup>4</sup> Only in the second and fourth matings is there any opportunity for crossing over, and in those two a total of only seventeen birds that would be affected by such crossing over.



Pink ♂	bM bM
Black ♀	BM bm
	BM bM — black ♂ 30
	bM bm — pink ♀ 33
	black ♀ 4

It is the last class of four black females which is of interest in this connection, and is inexplicable on the current scheme. I see only two explanations of this class—either the sire was, through some mistake, really black-eyed, which I mention only because it presents itself as a possible way out of the difficulty; or else, as I think probable, we have here an example of partial sex-linkage, *B* ordinarily being coupled with *M*. If this coupling be incomplete, then black females are to be expected from the last mating, as the following analysis will show:

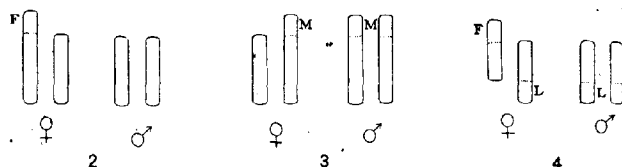
Gametes of pink ♂	bM bM
Gametes of black ♀	BM bm (Bm bM)
	BM bM — black ♂
	bm bM — pink ♀
	(bM bM — pink ♂)
	(Bm bM — black ♀)

This hypothesis could be easily tested. If it is correct, then the cross just discussed should, if large enough numbers be reared, produce as many pink males as black females. Furthermore, if these black females be bred to pink males, there should arise a race which would be dimorphic—black-eyed females and pink-eyed males—except for the occasional ‘crossing’ back of *B*, which would now be coupled with *m*, and *m* occurs only in the female. Such a relation, if it be shown to exist, would be highly interesting in its bearing upon the problem of secondary sexual characters.

The third case which I have interpreted as partial sex-linkage is that of the moth *Agria tau* reported by Standfuss ('96), and discussed by Castle ('03). The variety *lugens* of this species is dominant to the type. Its gene may be designated *L*. My interpretation of this case is that *L* and *M* are associated in such degree that ‘crossing over’ occurs in about one-third, instead of the usual one-half of the cases. The analysis of the matings



The cytological evidence relating to birds and Lepidoptera is not very helpful. Guyer's ('09, '09 a) reports on guinea-fowls and chickens are directly opposed to the experimental evidence, in that they make the male heterozygous for sex. \* Since a re-examination of the fowl case by other cytologists has so far failed to convince them that Guyer's view is correct, I think we may for the present disregard this evidence, at least in so far as it concerns the fowl. Several observers (see Stevens, '06; Dederer, '07; Cook, '10; Doncaster, '11) have studied the spermatogenesis of Lepidoptera, and in some cases have seen what they suspected to be an equal pair of idiochromosomes. I do not know of any further cytological evidence in these two classes of animals.



The cytological evidence indicates that, in the Lepidoptera at least, the male has two equal idiochromosomes. Judging by the experimental evidence, this must also be the case in birds, and the female must, of course, have at least one similar chromosome (see figs. 2 to 4). These three are the carriers of the genes for sex-linked factors. The doubtful point is the mate of this chromosome, present in the female-producing egg. If the sex formula be  $Ff$ ,  $ff$ , as we have been supposing, then this chromosome would be, visibly or imperceptibly, larger than the 'male' ( $f$ -bearing) chromosome, since it would have one factor,  $F$ , not present in that chromosome (fig. 2). In this case it would seem that complete sex-linkage, such as that found in *Abraxas* and in barred fowls, would occur not at all, or at least only rarely, since every part of the  $f$ -chromosome would have a homologous part in the  $F$ -chromosome, and crossing over would thus be possible. It might be that the process of reduction is such that no crossing over is possible in oogenesis, but if the cases of par-

tial sex-linkage be admitted, such crossing over must be possible.<sup>6</sup> If, on the other hand, we use the  $MM$ ,  $Mm$  scheme we meet with no such difficulties. In this case the heterochromosome contained in the female-producing egg is smaller than its mate in the male-producing egg, since it lacks the factor  $M$  (fig. 3),<sup>7</sup> and the sex-formula is  $MM$ ,  $Mm$ , two  $F$ 's being always present in both sexes, and contained, presumably, in some other pair of chromosomes. This would allow complete sex-linkage, for genes in that part of the  $M$ -chromosome having no homologue in the  $m$ -chromosome, and incomplete sex-linkage, for genes in the part having such a homologue. One might, of course evade this conclusion by assuming the condition represented in fig. 4. In this case the chromosomes would not conjugate evenly, and the part marked  $L$  could carry such factors as are completely sex-linked.

Of the converse case, where the male is heterozygous for sex there are not so many examples known. In *Drosophila* Morgan ('10, '11, '11 a, etc.) has reported numerous sex-linked factors. Miss Stevens ('08) has found here exactly the cytological conditions demanded by the experimental evidence. In man color-blindness follows this same scheme of inheritance, as do apparently several diseases (Bateson, '09; Morgan, '11). Here, too, Guyer ('10) has reported cytological evidence that it is the male which is heterozygous. Finally we have the case of partial sex-linkage referred to above. Miss Stevens ('11) has reported heterochromosomes in the male guinea-pig, and as that animal has been experimentally bred quite extensively I was led to look for sex-linkage in it. Perhaps the dwarf form studied by Miss Sollas ('09) is such a case. This is a recessive form, and has not been reared to maturity, or had not been in 1909, so that the case is not thoroughly worked out, but it seems to be most easily explainable as a case of partial sex-linkage. If we rep-

<sup>6</sup> It should be noted that, if my view is correct, the sex chromosomes under discussion are homologues, not of the  $X$  and  $Y$  of Diptera, Hemiptera, etc., but of the  $M$ -bearing chromosomes in those groups.

<sup>7</sup> On this scheme two  $F$ 's are to be assumed to be always present in both sexes, probably situated in another pair of chromosomes.

resent the factor for normal size, carried by the heterochromosomes, by  $N$ , then in ordinary guinea-pigs this is present in both gametes of each sex. But in a certain strain used by Miss Sollas, from which all her dwarfs descended, it had dropped out of the odd chromosome. This strain would go on breeding true, then, for some time, according to this scheme:

NF NF	normal ♀
NF nf	normal ♂
NF NF	normal ♀
NF nf	normal ♂

But now suppose crossing over sometimes occurred, and we should have:

	NF NF	normal ♀
NF nf	(nF Nf)	normal ♂ (gametes)
	NF NF	normal ♀
	NF nf	normal ♂
	(NF nF)	normal ♀
	(NF Nf)	normal ♂

We should still have no dwarfs, but if the heterozygous female were mated to a male of her own race we should get dwarfs, thus:

NF nF	normal ♀
NF nf	normal ♂
NF NF	normal ♀
NF nF	normal ♂
NF nf	normal ♂
nF nf	dwarf ♂

This explains why Miss Sollas should obtain a great majority of male dwarfs, and how, as seems to have occurred, the peculiarity may descend through the female. But she does get some dwarf females from normal parents. These are to be expected, since they would appear in the last mating above if crossing over again occurred in the male, thus:

	NF nF	normal ♀
NF nf	(nF Nf)	normal ♂ (gametes)
	NF NF	normal ♀
	NF nF	normal ♀
	NF nf	normal ♂
	nF nf	dwarf ♂
	(NF nF)	normal ♀)
	(nF nF)	dwarf ♀)
	(NF Nf)	normal ♂)
	(nF Nf)	normal ♂)

The actual numbers for families containing dwarfs is as follows:

Normal ♀	normal ♂	dwarf ♀	dwarf ♂
25	49	5	20

This gives a preponderance of normal males as against dwarf males, but it should be remembered that for every dwarf female due to crossing over there is a normal male produced, and secondly, the mutant is obviously not a very viable one, so that a shortage is perhaps not surprising, especially in such comparatively small numbers. The fact that there are nearly as many dwarf males as normal females is obviously due to the circumstance that the males of both kinds taken together are more than twice as numerous as the females.

In plants Correns ('07) and Shull ('10, '11) have shown that in certain dioecious species of *Bryonia* and *Lychnis* it is the male which is heterozygous. In the absence of cytological evidence or sex-linkage phenomena a chromosome interpretation of these cases would perhaps be out of place.

#### SUMMARY

There is a sex-linked factor carried by the Columbian Wyandotte—an inhibitor for red in the plumage. This breed probably also carries another sex-linked factor, an inhibitor for red in the neck. It apparently carries a pattern factor inhibiting the breast color, and, in the female, the stippled back of the Brown Leghorn.

The silver gray color is probably epistatic to the Jungle fowl or brown color.

The white Wyandotte is a silver laced breed with a color producer dropped out.

An attempt is made to explain three sets of phenomena, in fowls, in canaries, and in *Agla tau* respectively, as cases of partial sex-linkage. Using this explanation, it is argued that the sex formula for birds and *Lepidoptera* is probably; ♂, *MM*, *FF*; ♀, *Mm*, *FF*. The case of the dwarf guinea-pig is explained as perhaps representing partial sex-linkage in a form where the male is heterozygous for sex.

February, 1912

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# STUDIES ON THE PHYSIOLOGICAL CHARACTERS OF SPECIES

## I. THE EFFECTS OF CARBON DIOXIDE ON VARIOUS PROTOZOA

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### CONTENTS

I. Introduction.....	519
II. Material and methods.....	521
III. Observations and experiments.....	524
A. Ciliates.....	524
1. <i>Paramecium caudatum</i> .....	524
2. <i>Paramecium aurelia</i> .....	527
3. <i>Paramecium bursaria</i> .....	528
4. <i>Colpidium colpoda</i> .....	529
5. <i>Coleps hirtus</i> .....	530
6. <i>Blepharisma lateritia</i> .....	530
7. <i>Euplotes patella</i> .....	531
8. <i>Vorticella nebulifera</i> .....	532
B. Flagellates.....	534
1. <i>Peranema trichophorum</i> .....	534
2. <i>Euglena viridis</i> (?).....	535
3. <i>Chilomonas paramecium</i> .....	536
4. <i>Entosiphon sulcatum</i> .....	537
IV. Discussion of results.....	537
V. Summary.....	540
Bibliography.....	541

### I. INTRODUCTION

Considering its importance in connection with many aspects of modern biological research, the question of the physiological characters of species, as opposed to their morphological ones, has received a surprisingly small amount of attention. This condition has doubtless resulted partly from the fact that physiological characters, on account of their less definite and tangible

nature, are more difficult to deal with than morphological ones, and partly from the fact that single physiological characters at least, are notoriously unreliable guides in the questions of classification and phylogeny that up until the present day have occupied so large a share of the attention of working biologists.

The latter objection, however, no longer holds today, at least to the same extent that it formerly did. Modern zoology is not so much interested in finding out what are the probable relationships of a given animal as in learning what it is, and especially what it *does*. This is the physiological point of view, which is uppermost in the minds of most biologists today. No data which throw light on what goes on in the living organism are any longer considered unimportant; indeed, they are coming to be recognized as a vital necessity. If our knowledge of comparative physiology were as complete as our knowledge of comparative morphology, for example, there is not a single one of the more modern developments of biological science that would not have its possibilities enormously extended. It is therefore a matter of increasingly great importance to accumulate accurate data on the physiological characters of organisms, to determine which ones are fundamental, and which accidental, which are constant in a given species, or larger group, and which vary in different individuals of the same species, or perhaps in the same individual at different times; in short to obtain as full and comprehensive a knowledge as possible of the physiological characters of organisms. Perhaps the day may come when it will be possible to define any species in physiological and chemical terms in the same way in which it is now defined in morphological ones, and when no description of an organism will be considered complete which does not include its chief physiological peculiarities along with its structural one. The biologists of that day will be able successfully to attack problems that for the present must remain untouched on account of lack of the proper kind of knowledge.

It is needless to state that many observations of the sort suggested have already been made. Not to mention the more or less scattered ones made on many widely separated groups of organisms, we already have a considerable knowledge of the

physiological characters of many of the bacteria, a group in which, for obvious reasons, our physiological knowledge has far outstripped our morphological knowledge. Botanists have also accumulated an enormous fund of knowledge relating to the comparative physiology of the green plants, while in such special fields as the study of the blood sera of the higher vertebrates, to give but one example, encouraging progress has been made. Nevertheless, it is apparent that very little has been done in the way of systematic studies along the lines suggested, with the ultimate object of making the physiological characters of each organism as well known as its morphological ones. Such an undertaking is not the work of one man or of one generation. Many years must elapse before our knowledge will be anything but exceedingly fragmentary and scattered. The following paper is therefore a very modest contribution to so large a subject. It deals merely with the effects of a single common and important substance, carbon dioxide, on a number selected protozoan forms, with especial reference to their movements and general vitality. It will be followed at intervals by other papers on the effects of various other substances, so far as possible on the same forms. It is not claimed that the results are, or will be, complete or exhaustive; still it is hoped that they may not be without interest and a certain amount of value.

## II. MATERIAL AND METHODS

The forms studied were various of the most common ciliate Infusoria and flagellates, i.e., *Paramecium caudatum*, *P. aurelia*, *P. bursaria*, *Colpidium colpoda*, *Coleps hirtus*, *Blattarisma lateritia*, *Euplotes patella*, *Vorticella nebulifera*, *Peranema trichophorum*, *Euglena viridis* (?), *Chilomonas paramecium*, and *Entosiphon sulcatum*. In the case of all the forms mentioned except the last one, observations were made on individuals from several different cultures of different origin, the intention being to obtain, so far as possible, data which would apply to the species as a whole and not simply to a particular race. Of course it will be necessary to extend the observations still further before draw-

ing absolutely final conclusions; it is thought, however, that further work will not materially alter the results arrived at in this paper.

The general method employed in studying the effects of carbon dioxide on the forms in question was to subject them, in a drop of culture fluid, to a continuous stream of this gas in an Engelmann gas chamber. The drop of liquid containing them was placed on a slide or cover glass and the latter inverted in the usual way over the opening of the gas chamber, the joints being made airtight with vaseline. The observations were made entirely with the compound microscope, chiefly with a Leitz 3-objective, although in doubtful cases the 7-objective was also employed. The points especially noted were the time required to stop normal locomotion, the time required completely to stop the beat of the cilia, flagella, etc., and the longest possible exposure after which recovery is possible when normal conditions are restored. In addition, incidental observations were made on the general behavior of the organisms and the visible structural changes produced in the cell by carbon dioxide.

The gas used in the experiments was generated in the apparatus designed by McCoy, from marble and C. P. hydrochloric acid diluted in the proportion of one part of acid to four of water. Before coming in contact with the animals it was passed through two wash bottles filled with a solution of sodium carbonate to remove any traces of hydrochloric acid that might be present and also to ensure thorough saturation with water vapor. That no appreciable amount of hydrochloric acid was left in the gas was shown by conducting it into a silver nitrate solution, which in the course of two hours showed no traces of a precipitate or even of a cloudiness. The gas after being thus purified was conducted successively through four Engelmann chambers, each placed on the stage of a microscope, and connected by rubber tubing in such a way that the same gas passed through all of them. This arrangement was found very useful, not only in making comparisons between different species under as nearly identical conditions as possible, but also in facilitating a larger number of independent observations on individuals belonging to the same species.

Preliminary experiments showed the necessity of observing a number of precautions. The first of these is that the rate of evolution of the carbon dioxide gas shall be approximately the same in different experiments which it is desired to compare, since it was found that, other things being equal, the slower the stream of carbon dioxide passing over the drop the longer the animals survive. This is probably due to the fact that in a rapid stream the air is removed from the gas chamber and the drop more quickly, and the animals have less time to adjust themselves to the new conditions than in the case of a slow stream. By using all four of the gas chambers in one experiment it was found easy to compare a considerable number of forms with this factor constant. Frequently, indeed, a number of forms were present in the same drop and thus subjected to exactly the same conditions. In order to be able to compare experiments made on different days the attempt was made always to have the gas evolved at the rate of approximately 100 cc. per minute. This it was found possible to do within the necessary degree of accuracy by proper regulation at the beginning of the experiment of the apparatus, which is automatic when once started.

A second and most important point to be considered is the temperature, which has a marked effect on the time in which death occurs. A preliminary experiment on the three species of *Paramecium* showed that at 22°C. death occurs in roughly half the time in which it does at 12°C. In order that this factor might be made constant, all of the experiments here recorded were made at, or very near, the first mentioned temperature, which is slightly above ordinary room temperature.

A third point that cannot be neglected is the size of the drop containing the animals. Preliminary experiments showed that this has an appreciable effect on the results obtained, especially when the drop is very small. In one such experiment in a very small drop the average time of death of a certain 'pure' race of *Paramecium aurelia* was seventeen minutes while the average for the same race in a rather large drop was thirty minutes. In two medium sized drops in the same experiment the times were twenty-eight and twenty-nine minutes respectively. It will be

seen therefore that while there is not very much difference between the medium sized drops and the large one, the small one shows a decided difference. This is probably due to the greater suddenness with which the animals were subjected to the carbon dioxide in the latter case rather than to any difference in its concentration ultimately in the drops, since these probably all became practically fully saturated long before the end of the experiment. In order to guard against this source of error the drops were all made as nearly the same size as possible, the standard being a drop about 8 mm. in diameter with a moderate curvature. Results obtained in this way were constantly compared with those obtained when a number of the forms in question were present in the same drop.

The final precaution has already been mentioned, namely, not to base far-reaching conclusions on results obtained from a single culture. In the case of some of the forms studied more than a dozen cultures were employed; in all of them except one form, obtained very late, the number of cultures was at least three.

### III. OBSERVATIONS AND EXPERIMENTS

#### A. *Ciliates*

1. *Paramecium caudatum*. The effects of carbon dioxide on this form are briefly as follows. Immediately after the current of gas has been turned on, the animals exhibit a general restlessness and begin to seek the center of the drop, or rather, to avoid its edges, doubtless because the concentration of the carbon dioxide is greatest there. Inside a minute, as a rule, in a drop of the size used in these experiments, they have collected in its central and thickest part. Here they swim about actively but in very short paths, since the 'avoiding reaction' occurs whenever their movements have a tendency to carry them into the thinner and consequently more saturated part of the drop. Soon, however, generally within two or three minutes, they cease to be able to discriminate between the concentrations in different parts of the drop and spread out again until they are uniformly distributed. Sometimes toward the end of the experiment, for unknown reasons, they collect about its edge. Previous workers have noticed the

behavior just described. Loeb and Hardesty ('95) mention that *Paramecium aurelia* remains in the center of the drop fifteen minutes. In all probability they were dealing with a larger drop than the one used in these experiments, where in no case did either *P. aurelia* or *P. caudatum* require more than a few minutes to become adjusted to the new condition.

After this primary response, the animals swim about in a more or less normal manner but more and more slowly, until they finally come to rest in a time that may vary from twenty or twenty-five minutes to several hours. Even after locomotion has ceased the cilia continue to beat for some time. As death approaches they beat slowly and irregularly and frequently show visible signs of injury. Often a group of them may keep on beating after the others have come to rest. There seems to be nothing constant about the part of the body where movements persist longest. After the cilia have completely stopped it seems to be impossible to start them again even by prolonged exposure to the air. The animals are to all intents and purposes dead. The same thing is true of the other ciliates studied, with the exception of *Vorticella*, whose membranelles are often stopped before the animal is seriously injured and consequently can be started again. In the majority of ciliates, however, the vibratile structures are among the most resistant parts of the cell and when they have finally succumbed the life of the rest of the cell is practically extinct.

In the meantime, certain other changes have been occurring. Among the most striking of these is the change in shape of the body, which becomes shorter and thicker. Some of the increase in the thickness is doubtless due to the shortening, but this does not account for all of it, and it is probable that an actual increase in volume occurs by the absorption of water. This phenomenon is more strikingly shown in some of the other forms studied than in *P. caudatum*. About the time that the swelling becomes noticeable, the nuclei begin to be very clearly visible, standing out sharply from the rest of the protoplasm by their greater opaqueness. This is perhaps due to the acid nature of the medium surrounding the cell since other acids produce the same phenomenon. A further effect of the carbon dioxide often is apparent in the burst-



ing of the pellicle and the flowing out of droplets of clear protoplasm. This may occur either before or after the cilia have stopped beating, but is not so constant in *P. caudatum* as in some of the other forms studied. There is reason to believe that this result is at least partly due to actual injury of the pellicle and not merely to an increase of internal pressure, since the bursting sometimes occurs when the cell has not markedly swollen and also never appears until late, while the cell may reach almost its maximum volume quite early. Furthermore, after one droplet has formed, thus supposedly relieving the internal pressure, others may form in quick succession at other parts of the cell boundary. This effect is not a specific one of carbon dioxide since Budgett ('98) noted the same phenomenon in *Paramecium* and other protozoa when merely deprived of oxygen in a stream of hydrogen, and high temperatures also cause the same result.

The time that elapses from the beginning of the experiment until the death of the animal varies considerably with circumstances. The lowest average obtained in a single experiment was about twenty minutes, the highest over three-and-a-half hours, more cultures, however, approaching the latter value than the former. In an effort to determine to what extent *P. caudatum* can be said to have a specific resistance especial attention was paid to the question of the amount of variation shown by different individuals, races and cultures. A large mass of data was accumulated which will be made the basis of another paper on a somewhat different subject. It may not be out of place, however, to say here that while there is some evidence that different races may have different powers of resistance, these differences are insignificant compared with the enormous changes in resistance that a single race may undergo under appropriate changes in the culture medium. It is possible artificially to change the resistance very greatly, and such changes also occur naturally during the ageing of the culture, an old culture in general having a high resistance, and animals kept in the laboratory for a time being more resistant than 'wild' ones. Great as these variations are, however, they have their limits and in the dozens of cultures and thousands of individuals studied none were found which had as low a resist-

ance as the average *Coleps hirtus* for instance, or as high a one as the average *Colpidium colpoda*. Furthermore in a given culture, if *P. caudatum* has a higher resistance than usual, the other forms present also will, and their relative resistance remains practically constant. It is possible, therefore, to attribute to *P. caudatum* as well as to the other forms a specific resistance, remembering only that its absolute value is somewhat subject to variation under different conditions and that in comparing different forms it is well to have them either from the same culture or at least to make a considerable number of independent observations on different cultures.

2. *Paramecium aurelia*. A comparison of this form with the preceding one will illustrate the statement just made. *P. aurelia* is in general considerably less resistant than *P. caudatum*. When the two forms are present in the same culture the former is always killed sooner than the latter, though rarely, when different cultures are studied, some strains of *P. aurelia* are encountered which show a higher resistance than some of the most susceptible strains of *P. caudatum*. In general, however, *P. aurelia* is killed in less than a half hour while *P. caudatum* nearly always survives several times as long. The average time of death in the two extreme experiments on *P. aurelia* was a little over ten minutes on the one hand and over two hours on the other, in most of the experiments, however, lying, as already stated, below thirty minutes. Loeb and Hardesty state that *P. aurelia* is killed by carbon dioxide in two-and-a-half to three-and-a-half hours. Perhaps their culture was an abnormally resistant one, or possibly the application of the gas was slower than in these experiments, or the temperature lower. In view of the fact, however, that the figures given by them are quite typical of the rather more common *P. caudatum* at ordinary room temperature, and also that the distinction between *caudatum* and *aurelia* formerly was not very sharply drawn, it is possible that they were dealing with the former rather than the latter species. The difference in size between the two forms is probably not the reason for their different powers or resistance, since in the same species no constant relation could be found between the time of death and the size of the animal. Further-

more, the larger *P. bursaria* is *less* resistant than *P. aurelia* and the somewhat smaller *Colpidium colpoda* far more resistant. The cause of the difference is evidently of a more deep seated nature.

In the general effects produced on it by carbon dioxide, *P. aurelia* closely resembles the preceding species. It shows the same negative response at first, which disappears in about the same time. The body swells in about the same way. The nuclei often become very distinct, and this fact frequently renders an accurate identification possible without staining. The interval between the time when locomotion ceases and the cilia stop beating is both relatively and absolutely shorter than in *P. caudatum*. There is also a much greater tendency for the pellicle to rupture, this occurring in some cultures in almost every individual. Perhaps this apparently greater delicacy of the body wall may be correlated with the lower powers of resistance of this form.

3. *Paramecium bursaria*. In a number of the cultures used in these experiments this species was found associated with the two preceding ones and therefore a favorable opportunity was presented to compare it with them. Such a comparison shows that it is the least resistant of the three. The average time of death was generally ten to twenty minutes, though in a number of cultures it was less than five and only rarely ran as high as thirty. The most resistant *individual* found lived over an hour but this was a most exceptional case. (It was in this culture that *P. aurelia* also showed its highest resistance—over two hours.) When the three forms in question are present in the same culture in every case observed the relative resistance was: *bursaria*, *aurelia*, *caudatum*, and might perhaps be represented numerically very roughly as 1:2:4. The general effects of the carbon dioxide on this form are on the whole similar to those already described in the case of the other two species. The pellicle apparently is very delicate and nearly always ruptures while the cilia are more markedly affected than those of the other species; as their movements cease they become matted together and very quickly become partly disintegrated, being represented only by an indistinct zone about the animal. It is rather interesting that this green form is less resistant than the colorless ones. Doubtless

on account of the presence of chlorophyll in its body it is accustomed to rather a low concentration of carbon dioxide, since this substance is constantly being removed by it from the surrounding medium. Experiments to determine whether it was more, or less, resistant in bright light than in the dark have as yet not given very positive results, chiefly on account of the difficulty of controlling the temperature factor.

4. *Colpidium colpoda*. This in every case proved to be the most resistant species studied, living long after the other forms in the same drop had succumbed. The general effects on it of the carbon dioxide are as follows. When the stream of gas is turned on a strong negative response is shown and the animals collect in the center of the drop in the same manner as the forms already mentioned; soon, however, they become uniformly distributed and thereafter behave normally almost to the time of death which hardly ever occurs in less than six hours, and may take place much later. In a number of experiments the carbon dioxide was allowed to flow for six or seven hours and was then shut off without however admitting the air, and the animals were found to be in a normal condition the next day. In one such experiment they remained alive for a week, but this experiment was somewhat vitiated by the fact that a small quantity of chlorophyll derived from disintegrated *Englena* cells was present in the drop, and during the hours of daylight could have furnished a certain amount of oxygen, though only a small portion of the carbon dioxide in the chamber could have been gotten rid of in this way. However, even if these last results be discarded the fact remains that *Colpidium* is exceedingly resistant to carbon dioxide and may remain alive in a drop saturated with it for many hours. Prowazek ('03) found in an experiment of a different sort that *Colpidium* survives a simple lack of oxygen far better than *Paramecium caudatum*.

Carbon dioxide is not without its effects on *Colpidium*, however. Locomotion, while not so markedly affected as in most of the other forms, nevertheless becomes less active than normally. Before death occurs the animals become quiescent, often at the edge of the drop, and the cilia beat more and more slowly until they stop. One point of interest is that the animals, while they

move about in a fairly normal manner appear to take no food, and the food vacuoles present at the beginning of the experiment gradually are lost until the bodies of the organisms become remarkably transparent. For a long time there is no swelling of the body, but rather a narrowing, doubtless due to the loss of the food vacuoles; towards the last there may be a certain amount of swelling but the pellicle very rarely ruptures as it does in various species of *Paramecium*.

5. *Coleps hirtus*. This species forms a marked contrast with the preceding, being the least resistant of all the ciliates studied. Even the most resistant individuals hardly approach the least resistant ones of the other forms. The effect of the carbon dioxide is seen almost instantly. The animals show no decided negative reaction, apparently being overcome too quickly for such to occur. The normal, rather active movements of locomotion cease within a few seconds and thereafter only irregular 'vibrating' movements occur up to the time of death, of which the average is well under five minutes. Even the most resistant individuals do not live for ten minutes. The visible effects of the carbon dioxide are greater on this form than any of the others studied. The barrel-shaped body swells until it is broadly elliptical or even circular in outline, the increase in volume being very decided. At the same time the plates of the armature become indistinct and disappear, giving the appearance at least of actually being dissolved away. In consequence the body becomes very transparent and the protoplasm may be seen to undergo coagulation phenomena. The cilia sometimes beat after the armature has partly or entirely disappeared but usually their movements cease early. The cell in most cases bursts, generally at one of the ends; sometimes this occurs in two or three minutes, before much swelling has taken place.

6. *Blepharisma lateritia*. This on the whole is a very resistant form, being second only to *Colpidium* in this respect. It shows, however, rather more individual variation in the same culture than most of the other forms studied, isolated individuals sometimes succumbing quite early. Strangely enough, in spite of its general resistance its movements are very quickly affected. At

first it exhibits a slight negative response which, however, does not last very long and is not so decided as in the case of many of the other forms. Very soon its movements, never very active, become markedly slowed, and for long periods there is either no locomotion at all or this is very slow. The membranelles and cilia, however, keep on beating up to the time of death, which in the majority of cases occurs in from three to six hours. These two structures in *Blepharisma* seem to have about the same resistance. Two phenomena, which are particularly characteristic of this species are the marked change in form which occurs and the tendency towards the formation of large vacuoles in the protoplasm. The body, originally lanceolate, within an hour or two often becomes very broadly ovate or sometimes almost circular in outline. Corresponding to the general variability of the species in other respects, there is a great individual variation in this regard also. Towards the end, considerable distortion of the body occurs but bursting is rare.

7. *Euplotes patella*. This form, on account of its peculiar cuirass-like modification of the pellicle might be expected to show a high resistance, but such is not the case. Its resistance in general is somewhat below that of *Paramecium aurelia*. The effect of the carbon dioxide on it becomes apparent very early. It is not markedly stimulated, though it does show a negative response at first. The normal movements of locomotion disappear as a rule after five to ten minutes, although the membranelles sometimes keep on moving an hour longer. However, in most cases the average time of death is considerably less than this—generally under half-an-hour. The cell very early becomes much distorted and the pellicle of the ventral surface ruptures, allowing the escape of drops of clear protoplasm, while the macronucleus at the same time becomes very distinct and granular. Even after these changes have occurred, however, the membranelles continue their beat for a considerable time; the cirri are almost as resistant, though their movements are very irregular and uncoordinated towards the last. The average time required for all movements to cease varied in different experiments from about twenty-five minutes to a little over an hour. Rossbach

('72) claims that not only Euplotes but also Stylonychia and Chilodon and higher animals as well were completely killed by carbon dioxide in three minutes. Such low figures give grounds for the suspicion that his gas was not pure or that some other disturbing factor was present.

8. *Vorticella nebulifera*. This in many respects is the most interesting form studied. It presents a case where both vibratile structures (membranelles) and contractile ones (myonemes of the 'bell' and contractile filament of the stalk) are highly developed, and it is a point of considerable interest to compare the effect of carbon dioxide on these two classes of structures in the same cell. Its effects are as follows. Almost instantly when the gas is turned on the animal is strongly stimulated and makes perhaps half-a-dozen violent contractions of the stalk, which then slowly relaxes, and thereafter so long as the gas is allowed to flow neither contracts spontaneously nor can be made to do so by mechanical stimuli. The time required to produce this paralysis of the stalk varies from thirty to sixty seconds. The myonemes of the 'bell' are similarly affected and the animal remains fully expanded throughout the experiment. If the gas is not allowed to act too long, full and speedy recovery may occur on removal to the air. For instance, in one experiment after a five minutes' exposure to carbon dioxide and a subsequent five minutes' exposure to the air the animals were all normal in every respect. If the exposure to the gas is longer continued, however, permanent injury to the stalk results. After fifteen minutes the animals show a strong tendency to drop off of their stalks and the latter can be seen to be altered in appearance, the contractile filament becoming broken up into irregular refractive fragments and droplets. If the detached animals recover, they regenerate a new stalk.

The effect on the membranelles is almost the reverse of that on the contractile structures. At first they may show a temporary cessation of movement but usually inside a few moments they begin beating again and may continue to do so for three-quarters of an hour or more. Sometimes they stop temporarily and then start again even in the carbon dioxide while, removed to the air,

they show considerable powers of recovery after a lengthy period of rest. In this respect they differ from the membranelles of *Euplotes* and *Blepharisma* which in these experiments never could be made to resume their beat after having completely stopped. Doubtless this difference is due to the fact that in *Vorticella* they are accustomed to stopping every time the disc is retracted, while in the other two forms they normally remain in continuous motion and are stopped by nothing short of actual injury to the cell.

The powers of recovery of *Vorticella* after all movements have ceased are quite considerable. In one experiment after three-quarters of an hour practically all of the individuals had become quiet, and many had been in this condition for half-an-hour or more. Five hours after exposure to the air in a moist chamber about half of them had recovered and many had begun to regenerate the missing stalk, which was already one-tenth to one-half the length of the body. By the next day these stalks were one-half to two times the body length. In this experiment about 50 per cent of the individuals never recovered and this is typical of a number of experiments that were tried. It may be said, therefore, that certain of the movements of *Vorticella* are almost instantly affected and others only after a much longer time. Even after all visible movements have ceased the powers of recovery of the animals are considerable.

In a few instances individuals were observed which, before the beginning of the experiment, had formed the circle of cilia used in the free-swimming existence. Such cilia were about as resistant as the membranelles, though they were not observed to beat again after having stopped. In no case did individuals which broke from their stalks during the course of the experiment on account of the effect of the carbon dioxide, form such cilia, but their locomotion was entirely by means of their membranelles.

The *Vorticella* cell apparently shows no tendency to burst and form droplets of protoplasm, but a considerable change in form may occur. In an atmosphere of carbon dioxide *V. nebulifera* inside a few minutes tends to assume the more rounded form characteristic of *V. campanula*. In the individuals which are



killed the protoplasm becomes brownish and opaque, doubtless due to coagulation phenomena.

### *B. Flagellates*

1. *Peranema trichophorum*. This form shows conditions which suggest those already noted in *Vorticella*. As is well known, *Peranema* has a single very prominent flagellum, which in locomotion is directed straight forward. Ordinarily it is quite rigid except the tip, whose movements cause a slow forward progression of the animal. During progression, and also when otherwise at rest, the body shows very decided changes in form, these 'metabolic' movements, or contortions, being one of the most striking characteristics of the species. When exposed to carbon dioxide *Peranema* responds perhaps as quickly as any of the forms studied. Its forward progression ceases almost instantly and after a few preliminary, and rather vigorous contortions, the body suddenly becomes paralyzed in whatever state of contraction it may happen to be in at the time. Only rarely does the animal have time to contract to a spherical form before being overtaken with this paralysis; consequently the typical appearance of the body is an irregular mass which does not change its form so long as the stream of carbon dioxide is allowed to flow.

The flagellum responds differently. Contact with the carbon dioxide causes it to beat with a swinging motion in which the proximal as well as the distal portion is concerned. Sometimes these movements are rather vigorous, but they never give rise to locomotion. They may continue with more or less uniformity for a half or even three-quarters of an hour; at the end of that time the flagellum gradually comes to rest. All visible movements have now ceased, but the animals are not necessarily dead. Experiments were tried to determine what powers of recovery *Peranema* possesses after becoming perfectly motionless. It was found that when the drop containing them is removed from the gas chamber and placed in contact with the air, a considerable percentage of the individuals may recover after an exposure of an hour and a quarter to carbon dioxide, even though all movements have for a

long time ceased. In the case of individuals exposed for two hours, however, no recoveries occurred although the drop was kept under observation for twenty-four hours. The time required for recovery to occur depends on the length of time the gas has acted. After an exposure of five minutes, metabolic movements of the body begin in less than a half minute after contact with the air, and the animals may be entirely normal in ten minutes. After a longer exposure the time required is much greater. In one experiment after an exposure of thirty-four minutes, but few of the animals were in a state of normal activity after an hour-and-a-half in the air, though at the end of four hours most of them showed no signs of injury and were normal in every respect.

*Peranema* therefore represents a form in which the effects of carbon dioxide on locomotion and the contractile movements of the body are almost instantaneous, but which is killed only after a prolonged exposure. The point of greatest interest is that while certain movements of the body are brought to a standstill in a few seconds the flagellum may continue to beat for half-an-hour or more. We are therefore dealing with structures concerned in producing movements in the same cell which show a considerable physiological difference.

2. *Euglena viridis*(?). This form is in some respects more resistant and in others less resistant than *Peranema*. The time required for locomotion to cease is longer and the powers of recovery after an extended exposure greater, but the flagellum is much more sensitive and the length of time required to bring to an end all visible movements is considerably less. Like *Peranema*, *Euglena* shows no decided negative reaction to the gas as do many of the forms studied, though locomotion may persist for a few moments. Often the first effect of the gas is to cause a short temporary cessation of all movements, which quickly reappear. Soon, however, movements of progression cease and the organisms show signs of life only by vibrating movements which are due to the abnormal beat of the flagellum. These gradually cease and the animals sink to the bottom of the drop motionless and perfectly extended. The time required to produce cessation of all movement varies from two or three to ten minutes. The

particular *Euglena* studied, which was close to, but probably not identical with, *E. viridis*, was not one which very actively changes its form, and consequently was not a very favorable one in which to observe the effect of carbon dioxide on the contractile movements of the body. It may be said, however, that while 'euglenoid' movements were observed in many individuals before the beginning of the experiment and also after recovery, they never occurred during its progress, consequently the conditions here probably are the same, even if less striking, than those found in *Peranema*.

After the organisms have settled to the bottom of the drop and become motionless the only change that can be observed is a gradual slow swelling of the body. At the same time there is a slight shortening which, however, is not sufficient to account for the greater thickness of the organisms as careful measurements show. This swelling continues until the shape of the body has changed from cylindrical to broadly elliptical in outline and the chlorophyll bodies appear forced apart from each other. In extreme cases the cell may appear to be at the point of rupture, though this rarely occurs, the pellicle being very tough and elastic.

Although all movements cease in *Euglena* in ten minutes or less, it requires a much longer time to kill the organisms. Even after an exposure of three hours about a third of the individuals eventually recovered, though the time required was considerable. The recovery of *Euglena* is far slower than that of *Peranema*. After an exposure of seven minutes no recoveries could be noticed a half hour after removal to the air, although they began to occur soon after that, and in an hour and a quarter practically all the individuals were normal. After an exposure of two or three hours, the time required for recovery is three or four hours or more.

3. *Chilomonas paramecium*. This form shows great individual and also cultural variation. While in a few cases the animals become motionless in fifteen or twenty minutes, the average time required generally is three-quarters of an hour or more. Many resistant individuals retain their movements for several hours. In general, therefore, this may be said to be a form with a high resistance. Unlike the two previous flagellates, *Chilomonas*

shows most decided reactions to the presence of carbon dioxide. The first effect is often to cause a temporary cessation of motion which lasts however only for a few seconds, after which the animals are remarkably active. They show a striking tendency to seek the center of the drop at first, later becoming uniformly distributed again. Their motions at first are normal but gradually the animals come to rest and give evidences of life only by a slow rotation or by quick darting movements which they occasionally make. In practically every case the cell becomes circular in outline and if the experiment be long continued may actually burst. *Chilomonas* like the preceding form also has considerable powers of recovery after all motion has ceased. In one experiment even after an exposure to carbon dioxide of two-and-a-half hours about 75 per cent of the individuals eventually recovered after exposure to the air. In other cases, however, even after a shorter exposure the mortality is greater.

• 4. *Entosiphon sulcatum*. This form unfortunately was studied in only one experiment in which, however, a considerable number of individuals was present. Judging from these rather incomplete data, it is by far the most resistant of the flagellates examined. After an exposure of five hours it not only was alive but the movements were not very markedly affected. Both flagella continued to beat, though in rather a stiff and jerky fashion, and slow forward progression continued. How long it would have survived cannot be said, but probably the time would have been considerably above that mentioned, since when the experiment had to be ended none of the animals had as yet been killed.

#### IV. DISCUSSION OF RESULTS

From the results given it is apparent that all of the forms studied are injured and eventually killed by pure carbon dioxide, but that the resistance of the different forms is very different. *Colpidium colpoda* can withstand without injury an exposure of many hours, while *Coleps hirtus* is killed in three or four minutes. Sometimes the time of cessation of visible movements and the point at which the cell is so severely injured that recovery cannot occur, may coincide, as in most of the ciliates (in *Euplotes patella*

irreparable injury probably occurs before the membranelles cease beating) while in other cases, the animal is capable of full recovery long after all movements have ceased, e.g., flagellates and Vorticella. Some animals which are otherwise fairly resistant to carbon dioxide, as shown by their powers of recovery after a protracted exposure to it, or by the long continuation of visible movements, show its effects very quickly by their inability to carry on normal locomotion in its presence. *Paramecium* is the most striking example of this condition, *Euglena* and *Euplotes* also being relatively quickly affected. In their primary response the different forms also show distinct differences. The three species of *Paramecium* studied as well as *Colpidium*, and *Chilomonas* show a decided negative reaction and an effort to escape from it. This reaction is less marked in *Blepharisma* and *Euplotes*, while in the other forms it is practically lacking, probably because normal movements are so quickly interfered with. (*Entosiphon* was not studied in this connection.) It will be seen, therefore, that the different forms studied show certain characteristic differences in reactions and general resistance to carbon dioxide.

It has already been pointed out that there is a certain amount of individual and cultural variation in the same species, which makes it impossible to put in exact quantitative form the time in which death occurs, etc. Nevertheless the relative resistance of each form as compared with other forms from the same culture is fairly constant and furthermore it is at least possible to say that certain forms always have a high, and others always a low resistance. While some forms may 'overlap,' others, as for example *Colpidium* and *Coleps*, never do.

The observations here recorded are not the first that have been made on the differences in resistance to carbon dioxide shown by different organisms. Fränkel ('88) studied the effect of this substance on various bacteria with the result that some were found to thrive almost as well as in air, others had their development checked but were not killed, while others were quickly destroyed. Lopriore ('95) also, in his careful experiments on the effects of carbon dioxide on the spores and mycelia of fungi and the pollen

tubes of angiosperms, found decided specific differences. Many other scattered observations exist, which however, it is difficult to compare on account of the different methods employed in obtaining them.

It is interesting to consider the results here obtained in connection with those of Jennings and Moore ('01) on the chemotactic effect of carbon dioxide on various protozoa. Of the four forms mentioned by them as being attracted by this substance, three (i.e., *Paramecium caudatum*, *Colpidium colpoda*, and *Chilomonas paramecium*) have been studied in these experiments and all show marked powers of resistance, as well as a strong negative response when the concentration is suddenly made high in the edges of the drop. Of the forms found by them to be indifferent, unfortunately only two genera (*Euglena* and *Euplotes*) were available, but these both showed a relatively low resistance as compared with related forms, at least so far as the continuance of locomotion is concerned. It would be interesting to study the other members of their list in this connection. Doubtless many other facts of behavior could be brought into line with such physiological peculiarities as the one under consideration.

One of the most interesting results that appears from these experiments is the striking difference that seems to exist between the contractile elements of the cell—the myonemes—on the one hand, and the vibratile ones—cilia, membranelles, and flagella on the other. The former are very quickly thrown out of function while the latter continue their normal movements for a long time. The best illustration of this point is *Vorticella*, in which the contractile filament of the stalk, and the myonemes, after a primary stimulation, are inside a minute or less completely paralyzed, while the membranelles perhaps after stopping for a short time continue to beat for three-quarters of an hour. Such results are in agreement with those obtained by other workers. Neresheimer ('03), for example, found that the myonemes and membranelles of *Stentor* are differently affected by substances like morphin, which paralyze the former and do not affect the latter. Lillie ('12) has observed that the cilia of *Arenicola* larvae continue their activity for hours in isotonic sugar or magnesium chloride

solutions, or in solutions of chloroform or ether, which prevent completely all muscular movements. Recently Mayer ('11) has found that the effects of many ions on ciliary and muscular movements are exactly opposite those that depress the one stimulating the other. He found that the ciliary movements of trochophore larvae at first cease in water charged with carbon dioxide but later start again. It is well known that carbon dioxide at first stimulates and later depresses muscular movements in the higher vertebrates (Lee, '07). In *Vorticella* in the same cell this antagonistic action appears very clearly. In other forms it is harder to demonstrate a primary depression of ciliary activity, possibly because of the response of the organism as a whole in an adaptive way. In *Chilomonas*, however, the primary depression of the movements of the flagella nearly always occurs.

#### V. SUMMARY

1. Each of the twelve forms studied reacts to carbon dioxide in a characteristic way and has a characteristic resistance, which is highest in *Colpidium colpoda*, which remains alive many hours, and lowest in *Coleps hirtus*, which is killed in a few minutes. A certain amount of individual and cultural variation may occur which prevents the expression of the resistance of the species in absolute terms. Compared with other forms under the same conditions, however, the relative resistance is fairly constant.

2. Some forms are killed outright very quickly (*Coleps hirtus* and *Paramecium bursaria*). In others all movements are stopped in a few minutes but death occurs relatively late, the powers of recovery being high (*Euglena*). In still others, locomotion ceases very promptly but movements of the cilia, flagella, etc., may persist for a long time (*Peranema trichophorum*, *Euplotes patella*, etc.). In the remainder, more or less normal locomotion continues for a considerable time (most of the ciliates, *Chilomonas* and *Entosiphon*). The result in all cases, however, if the experiment be long enough continued, is cessation of movements and death.

3. In the same cell the contractile elements are usually quickly paralyzed (*Vorticella* and *Peranema*) while the vibratile structures (cilia, membranelles, flagella) are much more resistant. In some

cases (*Vorticella*) the action of carbon dioxide on these two classes of structures is exactly opposite, the contractile elements being first stimulated and then paralyzed and the vibratile ones often temporarily stopped and then started again.

4. Ordinary cilia and their modifications, membranelles and cirri when present in the same cell show approximately the same resistance. Flagella show great variation, that of *Euglena* being paralyzed in a few minutes and those of *Chilomonas* and *Entosiphon* remaining active for several hours.

5. In ciliates in general, with the exception of the specialized *Vorticella*, recovery after complete cessation of movement is impossible; in the flagellates, movement ceases long before the cell is permanently injured.

6. The general effects of carbon dioxide on the cell are to cause (a) cessation of movement, (b) absorption of water and consequent swelling, (c) injury to the cell wall, (d) death and coagulation of the protoplasm.

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## ON THE ADAPTATION OF FISH (FUNDULUS) TO HIGHER TEMPERATURES

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### I. INTRODUCTION

It is a well known fact that organisms can stand a higher temperature if the latter is raised gradually than if it is raised suddenly. This phenomenon is referred to in biology as a case of adaptation. Dallinger states that he succeeded in adapting certain protozoa to a temperature of 70° by gradually raising their temperature during several years.

Schottelius had found that colonies of *Micrococcus prodigiosus* when transferred from a temperature of 22° to that of 38° no longer formed pigment and trimethylamin. When transferred back to the temperature of 18° to 22° the formation of pigment and of trimethylamin was resumed. After the cocci had been cultivated for ten or fifteen generations at 38° they failed to form pigment even when transferred back to 22°.

These experiments became the starting point for similar experiments by Dieudonné. He used *Bacillus fluorescens putidus* which forms a fluorescein pigment and trimethylamin. The optimal temperature for this bacillus is 22°. At 35° it grew but did not form pigment or trimethylamin. At 37°.5 growth ceased. Re-transferred to 22° pigment and trimethylamin were again formed.

Dieudonné<sup>1</sup> exposed a culture of this bacillus to 35°. After twenty-four hours a second culture was taken from this and also kept at 35°, and this process was repeated each day. The fifteenth generation thus cultivated at 35° began to form some pigment and from the eighteenth generation on, at 35° the formation

<sup>1</sup> Dieudonné, Arb. aus dem kaiserl. Gesundheitsamte, vol. 9, p. 492, 1894.

of pigment and trimethylamin had become as good as that in the cultures kept at 22°. The organisms had, therefore, become 'adapted' to a temperature of 35° which at first was unfavorable.

Dieudonné did not succeed in causing pigment formation in this bacillus at a higher temperature than 35° although the bacillus finally grew at a temperature of 41°.5. He obtained similar results in experiments on other pigment-forming bacteria. Davenport and Castle<sup>2</sup> made experiments with tadpoles of frogs. One lot of eggs and tadpoles was kept at about 15°, a second lot for twenty-eight days at 25°C. While the tadpoles raised and kept at 15° went into heat rigor at 40°.3C., those kept for twenty-eight days at 25° were not affected by this temperature but went into heat rigor at 43°.5. Their resistance to high temperature had therefore risen 3°.2. When the latter tadpoles were put back for seventeen days to a temperature of 15° they had lost their resistance to high temperature to some extent but not completely, since they went into heat rigor at 41°.6. The authors suggest that this adaptation to a higher temperature is due to a loss of water on the part of the protoplasm. They assume that the rise in temperature causes a comparative acceleration in the excretion of water on the part of the tadpoles. The hypothesis of these authors is based upon the fact, that spores of bacteria are more resistant to high temperatures than the bacteria. While this is a fact, nothing in the experiments of Davenport and Castle proves that the amount of water in the tadpoles is diminished by the rise in temperature. The idea of Davenport and Castle was put to a direct test by Kryz.<sup>3</sup> He kept frogs, toads and salamanders at room temperature, and at temperatures as high as 40°C., for a number of days or weeks and tested the coagulation temperature of the muscle plasma for both ranges of temperatures. He found that the coagulation temperature was identical for the animals kept at low and those kept at high temperatures.

The following observations by Kammerer<sup>4</sup> indicate also an after-effect of the raising of temperature. *Lacerta muralis* from the

<sup>2</sup> Davenport and Castle, Arch. f. Entwicklungsmechanik, vol. 2, p. 227, 1896.

<sup>3</sup> Kryz, Arch. f. Entwicklungsmechanik, vol. 23, p. 560, 1907.

<sup>4</sup> Kammerer, Arch. f. Entwicklungsmechanik, vol. 30, p. 379, 1910.

cooler climate of Nieder Oesterreich darkens already at a temperature of 25° and becomes perfectly black at a slightly higher temperature. *Lacerta muralis* from the warmer climate of Italy remains perfectly normal at 25° and darkens only at 37°. Kammerer points out that the higher temperature at which the latter lizards had lived prevented the effects which the same rise in temperature produced in lizards that had previously lived at a lower temperature.

## II. THE INFLUENCE OF THE CONCENTRATION OF THE SOLUTION UPON THE RESISTANCE OF FUNDULUS TO HIGH TEMPERATURE

We used as material for our experiment half-grown *Fundulus* which were caught in Long Island Sound in January and kept in a room in the laboratory, the temperature of which varied a little around 10°C. Two fish were put into each of the following solutions: H<sub>2</sub>O, m/128, m/64, m/32, m/16, m/8, m/4, NaCl + KCl + CaCl<sub>2</sub> in the usual proportion. It was found that the higher the concentration of the solution—up to a certain limit—the longer the fish could survive in a given high temperature. The method consisted in putting a number of battery jars with 500 cc. of the solution into a large water bath the temperature of which was constantly watched and kept constant. When all the solutions had reached the desired temperature the fish were introduced. In a number of cases the experiment was continued for several days in a bacteriological thermostat. Table 1 gives the duration of life for these fish in each of the solutions for various tempera-

TABLE 1

TEMPERATURE	DURATION OF LIFE OF FUNDULUS IN								NaCl + KCl + CaCl <sub>2</sub>
	H <sub>2</sub> O	m/128	m/64	m/32	m/16	m/8	m/4	1 m	
25°	4 hrs.	indef.	indef.	indef.	indef.	indef.	indef.	indef.	
27°	1 hr.	2 hrs.	2 hrs.	indef.	indef.	indef.	indef.	indef.	
29°	7'	31'	50'	62'	90'	indef.	indef.	indef.	
31°	13'		25'	17'	50'	indef.	indef.	indef.	
33°	2'			6'	9'	7'	9'	80'	

tures. The term 'indefinite' means that the fish were alive and apparently normal at the end of the experiment.

These experiments were repeated with the same material with approximately identical results;  $33^{\circ}$  was as a rule the upper limit the fish could resist during the month of January. Occasionally a fish would survive a sudden exposure to a temperature of  $33^{\circ}$  in a  $m/4$  solution of  $\text{NaCl} + \text{KCl} + \text{CaCl}_2$  but such fish were no longer normal and would swim on their side.

It was next ascertained in which concentration the fish could resist the highest temperature. It was found that this concentration was  $m/4$ . In  $m/2$  and  $\frac{5}{8} m$  they were not able to resist as high a temperature as in  $m/4$  or  $\frac{3}{8} m$  solution of  $\text{NaCl} + \text{KCl} + \text{CaCl}_2$ . When sea-water was substituted for Ringer's solution ( $\text{NaCl} + \text{KCl} + \text{CaCl}_2$ ) the results were the same as represented in table 1.

TABLE 2

TEMPERATURE	DURATION OF LIFE OF FUNDULUS IN								DEX-TROSE
	$\text{H}_2\text{O}$	$m/32$	$m/16$	$m/8$	$m/4$	$\frac{3}{8} m$	$m/2$	$\frac{5}{8} m$	
$29^{\circ}$	40'	40'	30'	40'	100'	40'	70'	40'	
$31^{\circ}$	16'	20'	16'	20'	20'	24'	25'	18'	

The fact that the temperature which the fish could resist was the higher, the higher the concentration of the solution, suggested the possibility that the loss of water on the part of the fish increased their resistance. This explanation, however, does not agree with the observation made by Sumner and corroborated by us that *Fundulus* undergoes practically no change in weight when put into distilled water or when put back into sea-water. The idea that loss of water made *Fundulus* more resistant to heat could be tested by the substitution of sugar solution for Ringer's solution or sea-water. It was found that dextrose solutions were not able to protect the fish, in fact such solutions were little if any better than  $\text{H}_2\text{O}$  (table 2).

The duration of life of the fish in the dextrose solutions is about identical with that in distilled water. This excludes the suggestion that osmotic phenomena determine the influence of the con-

centration found in the experiments with Ringer solution or with sea-water; especially if we consider the fact that Loeb found that *Fundulus* can live for over a month in an  $m/8$  solution of dextrose at ordinary room temperature (if the solution is renewed every day).

The difference between the results expressed in tables 1 and 2 suggested that the protective action of the Ringer solution was of the nature of a specific salt action. We, therefore, tested the idea whether or not other salt solutions, *e.g.*,  $\text{NaCl}$  or  $\text{CaCl}_2$  could afford the same protection. It was found that this was true to a slight degree in the case of  $\text{NaCl}$  but not in the case of  $\text{CaCl}_2$ . Table 3 may serve as an example.

TABLE 3

TEMPERATURE	DURATION OF LIFE OF FUNDULUS IN							$\text{NaCl}$
	$\text{H}_2\text{O}$	$m/64$	$m/32$	$m/16$	$m/8$	$m/4$	$1\text{ }m$	
$29^\circ$	30'	30'	40'	50'	indef.	120'	120'	30'
$29^\circ$	$\text{H}_2\text{O}$	$m/64$	$m/32$	$m/16$	$m/8$	$m/4$	$1\text{ }m$	$\text{CaCl}_2$
	20'	100'	60'	90'	60'	30'	10'	

In an  $m/8$   $\text{NaCl}$  solution some but not all of the fish could live indefinitely at  $29^\circ$ ; in an  $m/8$  or  $m/4$  solution of sea-water or Ringer solution they could all live indefinitely at  $29^\circ$ . We must therefore conclude that the protection which sea-water or a Ringer solution gives *Fundulus* against a high temperature is due to a specific effect of the combination of the three salts  $\text{NaCl}$ ,  $\text{KCl}$  and  $\text{CaCl}$  in the right proportion. The idea presented itself that this protective action of the salts was the expression of an antagonism between the salts and a substance produced at a great velocity at a higher temperature, *e.g.*, an acid. Experiments, to be discussed a little further on, on the immunization of fish against a high temperature eliminate this possibility.

Experiments were tried on tadpoles and on a species of fresh water fish to ascertain whether these animals could resist high

A series of experiments was carried out in which fish were kept in a thermostat at 30°C. for various lengths of time to test whether this accelerated their adaptation to a temperature of 35°. This was true only to a slight extent. In all these experiments the fish were suddenly transferred to an m/4 Ringer solution at 35°.

We were curious to know if these animals could also survive if suddenly transferred to a temperature of 35° in distilled water. This is indeed the case as table 7 shows.

Fundulus can become adapted to a temperature of 35° in distilled water if they are kept for two days or longer at a temperature of 27°. It seemed to make no difference whether the fish had been kept at 27° in m/4 sea-water or in distilled water.

TABLE 7

PREVIOUSLY EXPOSED TO 27° FOR	DURATION OF LIFE OF FUNDULUS IF SUDDENLY PUT INTO DISTILLED WATER OF 35° C.
0 days (control)	4'
2 days	indefinite
3 days	indefinite

Finally experiments were made to see to how high a temperature these fish could be adapted in a week. By keeping the fish at a temperature of 27° over night and raising them during the day to a gradually higher temperature we found that they could be kept at the end of the week at a temperature of 40°C., for two hours without apparent injury. At a temperature of 41° they soon suffered in their power to maintain their equilibrium. They were immune to a temperature of 40° not only in an m/4 Ringer solution, but also in an m/64 solution. The lot which was in distilled water died early during the experiment through an accident. It is probable that Fundulus once adapted to a certain temperature can stand this temperature in any concentration of a Ringer solution below m/4.

## IV. THE SUMMATION OF THE IMMUNIZING EFFECTS OF SHORT PERIODS OF EXPOSURE TO HIGH TEMPERATURE

In the immunization experiments described thus far the fish had been exposed continuously for a rather long period of time to a temperature of 27°. We wanted to know if it was possible to immunize them for a higher temperature by exposing them only a short period of time each day and keeping them in the interval at a temperature of from 10° to 14°C. This would mean that the immunizing effect produced in the animal during a short exposure to a high temperature would be preserved at least twenty-four hours until the next exposure to a high temperature took

TABLE 8\*

DATE	DURATION AND TEMPERATURE OF EXPOSURE	
<i>March</i>		
7	1 hour from 17° to 31°	2 hours at 31°
8	1 hour from 17° to 33°	2 hours at 33°
9	1 hour from 18° to 35°	2 hours at 35°
11	3 hours from 11° to 37°	
12	2 hours from 17° to 37°	
13	2 hours from 16° to 37°	
14	2 hours from 17° to 37°	
15	2 hours from 17° to 37°	2 hours at 37°
16	2 hours from 17° to 37°	2 hours at 37°
18	2 hours from 17° to 38°	1½ hours at 38°
19	2 hours from 18° to 38°	2 hours at 38°
20	2 hours from 19° to 39°	2 hours at 39°

place; and would be added to the immunizing effect of the next exposure to a high temperature. Table 8 gives the periods of exposure.

Most of the fish died on the third day when the temperature was raised only to 35°. For this reason we did not dare to expose the fish for more than a few minutes to a temperature of 37° on the 11th, 12th, 13th and 14th of March. The fish were exposed to a higher temperature for not more than four hours on one day. We have seen that an exposure of four hours in itself does not suffice to create immunity to a temperature of 35° or above. Hence the fact that these fish were finally able to resist a



temperature of 39° indicates a cumulative effect of the different exposures to a higher temperature. In other words, each heating increased the immunity and this gain was not lost during one or two days.

V. THE IMMUNITY TO A HIGH TEMPERATURE IF ONCE ACQUIRED  
IS KEPT FOR MORE THAN FOUR WEEKS

In order to prove this, fish were put for various lengths of time into a thermostat at 27°, tested in regard to their immunity against high temperature and then put back into the cold room and tested again. A few examples will illustrate this. Five fish were immunized to a temperature of 39° by exposing them daily for a number of hours to an increasing temperature, until they could live in a temperature of 39°C. (in m/4 Ringer solution). The process of acclimatization extended over a period of twelve days (see previous experiment). After this they were kept for eight days constantly at a temperature of from 10° to 14°C. On the eleventh day they were put suddenly into a temperature of 31° and the temperature of the water in which they were, was brought, inside of two hours, to a temperature of 39°, and then kept at this height. A control experiment was carried on simultaneously with fish taken from the same cold room, which had not been acclimated. The solutions used were m/4 Ringer. The control fish that had not been acclimated to high temperature were dead in one and a half hour when the temperature had reached 36°. The acclimated fish kept alive for over an hour at 39° when the experiment was discontinued. In eight days, therefore, the immunity of the fish to high temperature had not diminished.

Four lots of fish had been immunized to a temperature of 35° by keeping them twenty-four, thirty-two, forty and seventy-two hours respectively at a temperature of 27°. After this the fish were put into the cold room and kept there at a temperature ranging from 10° to 15°C., for twenty-eight days. They were then put into an m/4 Ringer solution at a temperature of 35°. Simultaneously six fish of the same lot, which had not been immunized but kept in the cold room permanently, were put into the same temperature and the same solution. Four of the latter fish died

within two minutes, the rest were dead forty minutes later. The fish that had been immunized before were, with the exception of two individuals, all alive and normal after five hours. Yet, the fact that two of the fish succumbed may be an indication that their resistance to  $35^{\circ}$  was less than immediately after immunization. It is quite possible, however, that these two fish which had been kept in small dishes for such a long time had suffered through this captivity.

This idea is supported by the fact that in a third experiment fish had kept their immunity to high temperature for thirty-three days after immunization against  $35^{\circ}$ . The immunization consisted in exposing the fish for three and six days respectively to  $27^{\circ}$ . After that they were kept in the cold room for thirty-three days. When after that time subjected to a temperature of  $35^{\circ}$  they remained perfectly normal for five and a half hours, when the experiment was discontinued.

We made a large number of experiments in which the duration of the immunity against high temperatures was tested sooner after the process of immunization than in the above mentioned experiments. In all these experiments it was found that the fish did not lose their immunity against temperatures of  $39^{\circ}$  and  $35^{\circ}$  respectively if they were put into the cold room for a period of thirty-three days or less after immunization.

#### VI. EXPERIMENTS WITH FISH KEPT AT A CONSTANT TEMPERATURE OF $0.4^{\circ}$ C.

The fish which we used for this experiment were caught in January and kept since that time in a cold room in which the temperature varied between  $10^{\circ}$  and  $14^{\circ}$  C. Our experiments showed that these fish died in a rather short time when suddenly put into a diluted Ringer solution or diluted sea-water of  $31^{\circ}$ , provided that the concentration of the solution was below m/8. In an m/8 or m/4 Ringer solution or sea-water they were able to resist the temperature of  $31^{\circ}$  without any previous immunization. We put a large number of these fish in an ice chest in which the temperature remained constantly at  $0.4^{\circ}$ , and investigated at various intervals whether the resistance of these animals to a

temperature of 31° differed from that of the fish kept at from 10° to 14° (cold room). The fish put in the ice chest had previously been at a temperature of 10° for several weeks.

If we consider the behavior of the fish in an m/8 solution at 31° we notice a steady diminution of resistance among those kept at 0°.4; while among those kept at 10° to 14° the resistance increased somewhat. The latter result is not accidental. We must remember that the fish were taken in January when the temperature of the water was not far from 0°C. The long exposure to a temperature of from 10° to 14° had therefore a slight immunizing effect.

Our next task was to ascertain whether fish immunized to resist a sudden transfer to a temperature of 35° kept this immunity if put on ice just as well as if kept at a temperature of 10° for the same period. Our experiments thus far cover only an exposure of fourteen days on ice ( $T. = 0°.4$ ). During this time the immunity was not diminished, as the following example will show. Fish were immunized to a sudden transfer to 35°C. by keeping them for two days at 27°. They were then put into a thermostat with a constant temperature of 0°.4 for fourteen days and put directly

TABLE 9

KEPT AT 0°.4° C.	DURATION OF LIFE AT 31° IN				RINGER SOLUTION
	H <sub>2</sub> O	m/32	m/16	m/8	
7 days	26'		104'	indef.	
19 days	13'	42'	17'	some indef.	
33 days	24'	30'	69'	80'	
41 days	22'	34'	22'	8'	

KEPT AT 10° C.	DURATION OF LIFE AT 31° IN				RINGER SOLUTION
	H <sub>2</sub> O	m/32	m/16	m/8	
Over 7 days	20'		135'	indef.	
Over 19 days	13'	26'	52'	indef.	
Over 33 days	42'	86'	some indef.	indef.	
Over 41 days	41'	some indef.	some indef.	indef.	

into a m/4 Ringer solution of a temperature of  $35^{\circ}$  (A). Simultaneously fish which had been immunized for  $35^{\circ}$  by keeping them three days at  $27^{\circ}$  and which had then been kept at between  $10^{\circ}$  and  $14^{\circ}$  for nineteen days were also put into a m/4 Ringer solution of  $35^{\circ}$  (B). In addition two controls were made: Fish kept on ice at  $0^{\circ}$  for twenty days but not previously immunized (C), and fish not previously immunized kept for several weeks at a temperature of from  $10^{\circ}$  to  $14^{\circ}$  (D) were also suddenly transferred to a temperature of  $35^{\circ}$ . Table 10 gives the result.

TABLE 10

DURATION OF LIFE OF FISH AT  $35^{\circ}$  IN M/4 RINGER SOLUTION

A. Immunized but kept on ice for fourteen days	B. Immunized but kept at $10^{\circ}$ for nineteen days	C. Not immunized kept on ice for twenty days	D. Not immunized kept in cold room
Alive after 3 hours	One alive after 3 hours	Die in 2'	Die in 2'

The experiment was repeated with the same result, only those in lot A and B remained all alive. It is therefore obvious that the resistance acquired for a higher temperature ( $35^{\circ}$ ) is not lost or diminished if the fish are kept for two weeks on ice.

## THEORETICAL

The phenomenon of adaptation considered in this paper is the fact that fish can resist a high temperature better if the latter is raised gradually than when it is raised suddenly. Physics offers us an analogy to this phenomenon in the experience that glass vessels which burst easily when their temperature is raised suddenly, remain intact when the temperature is raised gradually. This phenomenon finds its explanation in the fact that glass is a poor conductor of heat and that when the temperature is raised suddenly, *e.g.*, inside a glass cylinder, the inner layer of the cylinder expands while the outer layer, on account of the slowness of the conduction of heat, does not expand equally and the cylinder bursts.

The following idea for the explanation of the mechanism of adaptation suggests itself. The rise in temperature brings about certain changes especially in the surface of the cells or the body of the animal, whereby the latter loses its protective impermeability. If the rise in temperature occurs gradually the blood (and especially the salts of the blood or of the surrounding solution or of both) has time to repair the damage. If the rise, however, occurs suddenly then the damage done cannot be repaired quickly enough by the blood, or the salts of the surrounding solution, to prevent the death of the cell or the animal. The peculiar influence of the concentration and nature of the surrounding solution described in this paper would harmonize with this suggestion.

A second possible suggestion is that under the influence of the higher temperature a substance is formed in the animal which protects it against the effects of high temperature. The formation of this substance is also a function of time and for this reason an animal can keep alive if the temperature is increased gradually but cannot keep alive if it is increased rapidly.

Both suggestions would explain the fact that if an animal is once immunized against a high temperature it will keep this immunization, for some time at least, even if kept at a low temperature or on ice. Further experiments with which we are occupied may decide between these and other possible suggestions.

#### SUMMARY

1. Experiments were made with *Fundulus* which were caught in the winter and kept at a low temperature (from 10° to 14°C.), to find out the maximum temperature into which they could, with impunity, be transferred suddenly. It was found that the maximum temperature varied with the concentration of the sea-water or a Ringer solution; being about 25°C. for a concentration of m/128 or m/64; 27°C. for a concentration of m/32; 31°C. for a concentration of m/8, and almost 33°C. for a concentration of m/4. The latter concentration was the optimum, the resistance to high temperature decreasing again with a further rise in concentration.

2. It was found that dextrose solutions were not able to afford any protection against the effects of a sudden rise in temperature. From these and similar experiments with  $\text{CaCl}_2$  solutions it follows that the protective action of sea-water or a Ringer solution against high temperature is not an osmotic but a specific effect of the salts of the sea-water.

3. It was ascertained how long it takes to immunize the fish against the harmful effects of a sudden transfer to a temperature of  $35^\circ\text{C}$ . It was found that by keeping the fish for thirty hours or more at a temperature of  $27^\circ$  they were immunized against a temperature of  $35^\circ$ . Often a noticeable immunizing effect was produced already by an exposure of sixteen hours or even a little less to a temperature of  $27^\circ$ . Fish kept for two days at  $27^\circ$  were able to survive if suddenly transferred to distilled water of  $35^\circ\text{C}$ .

4. The immunity against a temperature of  $35^\circ$  acquired by keeping the fish for two days at  $27^\circ$  is not lost or weakened if the fish are afterwards kept as long as thirty-three days at a temperature of from  $10^\circ$  to  $14^\circ$ . Our experiments have not been extended beyond this period of time.

5. The immunity against a temperature of  $35^\circ\text{C}$ . is also maintained if the fish are kept after the two days' exposure to  $27^\circ$  for two weeks at a temperature of  $0^\circ.4\text{ C}$ .

6. Fish immunized against a temperature of  $39^\circ$  and then kept at a temperature of from  $10^\circ$  to  $14^\circ$  for eleven days did not lose their immunity.

7. A longer exposure of fish to a temperature of  $0^\circ.4$  may finally lower their resistance to high temperature.

8. In order to immunize fish to a temperature of  $39^\circ$  it is not necessary to expose them continuously to a higher temperature. An intermittent exposure to a higher temperature during a number of hours each day will bring about the same effect.

9. Various suggestions for a possible theory of these phenomena are made.



## AUTHOR AND SUBJECT INDEX

- ADAPTATION** of fish (*Fundulus*) to higher temperatures. On the effects of alcohol, nicotine, tobacco smoke and caffeine on white mice. Comparative studies of the effects of I. Effects on reproduction and growth. 133
- Amphibians.** Studies on sex-determination in. V. The effects of changing the water content of the egg at or before the time of fertilization on the sex-ratio of *Bufo lentiginosus*. 319
- Arbacia-Toxopneustes crosses.** Studies in cytology. I. A further study of the chromosomes of *T. variegatus*. II. The behavior of the chromosomes in 391
- BANCROFT, FRANK W.** Heredity of pigmentation in *Fundulus* hybrids. 153
- BANCROFT, F. W., LOEB, JACQUES AND.** Can the spermatozoon develop outside the egg? 381
- CAFFEINE** on white mice. Comparative studies on the effects of alcohol, nicotine, tobacco smoke and I. Effects on reproduction and growth. 133
- Carbon dioxide** on various protozoa. Studies on the physiological characters of species. I. The effects of 519
- Cell size and nuclear size.** 1
- Characters of species.** Studied on the physiological. I. The effects of carbon dioxide on various protozoa. 519
- Chemical properties of hay infusions** with special reference to the titratable acidity and its relation to the protozoan sequence. 265
- Chromosomes of *Toxopneustes variegatus*.** II. The behavior of the chromosomes in *Arbacia-Toxopneustes* crosses. Studies in cytology. I. A further study of the 361
- CONKLIN, EDWIN G.** Cell size and nuclear size. 1
- COPPELAND, MAXTON.** The olfactory reactions of the puffer or swellfish, *Spheroides maculatus* (Bleek and Schnetler). 563
- CURTIS, MAYNIE R., PEARL, RAYMOND AND** Studies on the physiology of reproduction in the domestic fowl. V. Data regarding the physiology of the oviduct. 99
- Cytology.** Studies in I. A further study of the chromosomes of *Toxopneustes variegatus*. II. The behavior of the chromosomes in *Arbacia-Toxopneustes* crosses. 391
- DUCKS.** Size inheritance in 369
- FERTILIZATION** in *Hydratna senta*. Reinvigoration produced by cross 337
- Fertilization in *Nereis*.** Studies of III. The morphology of the normal fertilization. IV. The fertilization power of portions of the spermatozoon. 413
- FINE, MORRIS S.** Chemical properties of hay infusions with special reference to the titratable acidity and its relation to the protozoan sequence. 265
- Fowl.** Studies on the physiology of reproduction in the domestic. V. Data regarding the physiology of the oviduct. 99
- Fowls.** An experiment dealing with sex-linkage in 599
- Fundulus* hybrids. Heredity of pigmentation in 153
- Fundulus* to higher temperatures. On the adaptation of fish 543
- GROWTH.** Comparative studies on the effects of alcohol, nicotine, tobacco smoke and caffeine on white mice. I. Effects on reproduction and 133
- HEREDITY** of pigmentation in *Fundulus* hybrids. 153
- Hybrids.** Heredity of pigmentation in *Fundulus* 153
- Hydratna senta*.** Reinvigoration produced by cross fertilization in 337
- Hydratna senta*.** Studies in the life cycle of. III. Internal factors influencing the proportion of male-producers. 283
- INFUSIONS.** Observations on the origin and sequence of the protozoan fauna of hay. 265
- Infusions with special reference to the titratable acidity and its relation to the protozoan sequence.** Chemical properties of hay 265
- Inheritance in ducks.** Size 369
- Internal factors influencing the proportion of male-producers.** Studies in the life cycle of *Hydratna senta*. 283
- JACOBS, MERKEL HENRY.** Studies on the physiological characters of species. I. The effects of carbon dioxide on various protozoa. 519
- KING, HELEN DEAN.** Studies on sex-determination in *Amphibians*. V. The effects of changing the water content of the egg at or before the time of fertilization on the sex-ratio of *Bufo lentiginosus*. 319
- LIFE cycle of *Hydratna senta*.** Studies in the. III. Internal factors influencing the proportion of male-producers. 283
- LAURE, FRANK R.** Studies of fertilization in *Nereis*. III. The morphology of the normal fertilization of *Nereis*. IV. The fertilization power of portions of the spermatozoon. 413
- LOEB, JACQUES, and BANCROFT, F. W.** Can the spermatozoon develop outside the egg? 381
- LOEB, JACQUES, and WASTENHAY, HAROLD P.** On the adaptation of fish (*Fundulus*) to higher temperatures. 543
- Longevity in *Saturniid* moths; an experimental study.** 179
- MALE-PRODUCERS.** Studies in the life cycle of *Hydratna senta*. III. Internal factors influencing the proportion of 283
- Male-producing eggs of *Phylloxera*.** The elimination of the sex-chromosomes from the 479
- Mice.** Comparative studies on the effects of alcohol, nicotine, tobacco smoke and caffeine on white. I. Effects on reproduction and growth. 133
- MOREMAN, THOMAS H.** The elimination of the sex-chromosomes from the male-producing eggs of *Phylloxera*. 479
- Moths; an experimental study.** Longevity in *Saturniid* 179













